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Studies on immune regulation in the development of human type 1 diabetes

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Abstract

Type 1 diabetes is an autoimmune disease caused by T-cell-mediated destruction of the insulin-producing beta cells of the pancreas. The exact mechanisms leading to type 1 diabetes are still mostly unknown, but genetic and environmental factors are involved. This doctoral study aimed at characterizing novel immune responses associated with the development of type 1 diabetes.

We were interested in peripheral immunological responses in T helper cells. One environmental factor we focused on was vitamin D, since epidemiological data have associated it with type 1 diabetes and it has a direct effect on T cells. The aims of this study were to investigate the role of vitamin D during the fetal period for type 1 diabetes by analyzing first-trimester serum samples from mothers of healthy and diabetic children. A low concentration of serum 25-hydroxyvitamin D (25(OH)D) during the first trimester of pregnancy was not associated with type 1 diabetes in the offspring. Furthermore, we investigated the possible protective role of vitamin D in beta-cell autoimmunity in young children from Finland and Estonia,

two countries differing in the incidence of type 1 diabetes, standards of living, and vitamin D fortification of foods at the time of the study. We did not find differences in the vitamin D status or active hormone levels (1,25-dehyroxyvitamin D (1,25(OH)$_2$D)) of vitamin D between the study groups. Estonian children had lower 25(OH)D concentrations than Finnish children, but their 1,25(OH)$_2$D concentrations were at the same sufficient level. Our results suggest that vitamin D deficiency in the first trimester of pregnancy is not a risk factor for the offspring developing type 1 diabetes later in life. Our results from young children further suggest that a low peripheral vitamin D concentration is not associated with the development of beta-cell-associated autoimmunity.

Additionally, we investigated the timing of up-regulation of T helper (Th)17 immunity and the role of Th17 plasticity in the development of type 1 diabetes in prediabetic children. Stimulated peripheral blood mononuclear cells (PBMCs) from children with multiple beta-cell autoantibodies and impaired glucose tolerance revealed up-regulation of Th17 immunity and increased mRNA expression of interferon-γ (IFN-γ) and interleukin-9 (IL-9). These children also had higher IFN-γ mRNA expression in fluorescence-activated cell sorting (FACS)-purified Th17 cells. We observed that Th17 immunity and increased plasticity of Th17 cells is associated with advanced beta-cell autoimmunity and it correlated with clinical parameters.

Finally, we examined peripheral cytokine, chemokine, and growth factor profiles in healthy children from Russia, Estonia, and Finland, countries with different incidences of type 1 diabetes and standards of living. We observed that Finnish children had a lower concentration of epidermal growth factor (EGF) and soluble CD40 ligand (sCD40L) in their blood. We observed higher mRNA expression of interleukin-22 (IL-22) in circulating memory T helper cells of Estonian children. We also noted that Th17 and

Th1 immunity-related cytokines were up-regulated in the blood of Finnish children.

To conclude, vitamin D is not a direct protective factor in the development of beta-cell autoimmunity or type 1 diabetes. Th17 immunity and plasticity associate with type 1 diabetes disease progression from advanced beta-cell autoimmunity to clinical disease. These data may provide new tools for improved disease development monitoring and for selecting individuals at risk to be included in secondary intervention trials. The low EGF and sCD40L levels in the blood of Finnish children may modulate the epithelium of the intestine and hence result in altered T-cell differentiation, leading to pathogenic Th17 immunity. In the future, we should further focus on determining the relationship between vitamin D, EGF, and Th17 immunity, since EGF may regulate immune cell functions either directly or indirectly through the regulation of epithelial integrity, which in turn may affect the risk of developing type 1 diabetes.

Keywords: Type 1 diabetes, T cell, Vitamin D, Th17 immunity, Epidermal growth factor

Helsingin yliopisto, Lääketieteellinen tiedekunta
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Tiivistelmä

Tyypin 1 diabetes on autoimmuunisairaus, jossa elimistön T-solut aiheuttavat haiman insuliinia tuottavien beetasolujen tuhoutumista. Taudin syntymekanismit ovat edelleen suurelta osin tuntemattomia, mutta tiedetään, että sekä geneettiset että ympäristötekijät osallistuvat prosessiin. Tämän tutkimuksen tarkoituksena oli kuvata uusia sairauden kehittymiseen liittyviä immuunivasteita.

Tutkimuksessa keskityttiin perifeerisiin immuunivasteisiin T-auttajasoluja analysoimalla, sekä ympäristötekijöistä D-vitamiinin, jonka on ajateltu mahdollisesti suoajaavan taudilta. Yhtenä tavoitteena oli määrittää seeruminäytteistä D-vitamiinitasoja (seerumin 25-hydroksi-D-vitamiini; 25OHD) raskauden ensimmäisen kolmanneksen ajalta, ja tutkia, onko tasoilla merkitystä siihen, sairastuuko lapsi myöhemmin tyypin 1 diabetekseen. Alhainen seerumin 25(OH)D pitoisuus raskauden ensimmäisen kolmanneksen aikana ei liittynyt taudin kehittymiseen lapsilla. Halusimme

myös tutkia D-vitamiinin mahdollista suojaavaa vaikutusta beetasoluautoimmuniteetille alttiissa nuorissa suomalaisissa ja virolaisissa lapsissa. Näissä maissa tyypin 1 diabeteksen ilmaantuvuus, elintaso sekä elintarvikkeiden D-vitaminointi olivat tutkimuksen aikaan erilaisia. Emme kuitenkaan nähneet eroja ryhmien välillä, joilla oli eri riski sairastua tyypin 1 diabetekseen, D-vitamiinitasoissa tai aktiivisen hormonin (seerumin 1,25-dihydroksi-D-vitamiini; $1,25(OH)_2D$) tasoissa. Virolaisilla lapsilla oli selvästi matalammat D-vitamiinitasot, mutta heidän $1,25(OH)_2D$-pitoisuudet olivat samalla riittävällä tasolla kuin suomalaisilla lapsilla. Tuloksemme osoittavat, että D-vitamiinin puutos raskauden ensimmäisen kolmanneksen aikana ei ole riskitekijä tyypin 1 diabeteksen kehittymiselle lapsilla eikä D-vitamiinilla ole suojaavaa vaikutusta beetasoluautoimmuniteetin kehittymiselle nuorissa lapsissa.

Lisäksi halusimme selvittää aiemmin tyypin 1 diabetekseen liitetyn T-auttajasolupopulaation (Th17-solut) aktivoitumisen ajoitusta tautiprosessissa ja Th17-solujen plastisuuden merkitystä taudin kehittymisessä prediabeettisissa lapsissa. Osoitimme lisääntyneen Th17-immuniteetin stimuloiduissa perifeerisen veren mononukleaarisissa soluissa (PBMC) lapsilla, joilla oli useita beetasoluautovasta-aineita sekä heikentynyt sokerinsieto sokerirasituskokeessa. Näissä soluissa näkyi myös lisääntynyt interferoni-γ (IFN-γ) ja interleukiini-9:n (IL-9) mRNA ekspressio. Lisäksi havaitsimme samojen lasten virtaussytometrisesti puhdistetuista Th17-soluista lisääntyneen IFN-γ mRNA ekspression. Havaitsimme, että Th17-immuniteetti sekä -plastisuus liittyy pitkälle edenneeseen beetasoluautoimmuniteettiin.

Lopuksi halusimme tutkia veren sytokiini-, kemokiini- ja kasvutekijäprofiileja terveillä lapsilla Venäjältä, Virosta ja Suomesta, maista, joissa sekä tyypin 1 diabeteksen ilmaantuvuus että elintaso ovat erilaisia. Havait-

simme, että suomalaisilla lapsilla oli alhaisempi epidermaalisen kasvutekijän (EGF) ja liukoisen CD40-ligandin (sCD40L) pitoisuus veressä. Lisäksi havaitsimme korkeamman interleukiini-22 (IL-22) mRNA ekspression virolaisten lasten veren T-auttajamuistisoluissa. Huomasimme myös, että Th17- ja Th1-immuniteettiin liittyvät sytokiinit olivat suomalaisten lasten veressä koholla.

Tulosten perusteella voidaan todeta, että raskauden ensimmäisen kolmanneksen tai varhaislapsuuden D-vitamiinitasoilla ei ole suoraa yhteyttä beetasoluautoimmuniteetin tai tyypin 1 diabeteksen kehittymiseen. Th17-immuniteetti ja -plastisuus liittyvät taudin kehittymiseen ja täten näitä voitaisiin mahdollisesti käyttää työkaluina riskiyksilöiden valinnassa interventiotutkimuksiin. Suomalaisilla lapsilla havaitut matalat EGF- ja sCD40L-pitoisuudet saattavat aiheuttaa muutoksia suoliston limakalvolle ja näin ollen muuttaa T-auttajamuistisolujen profiilia patogeenisten Th17-solujen suuntaan. Tulevaisuudessa olisi kiinnostavaa selvittää edelleen D-vitamiinin, EGF:n ja Th17-immuniteetin välisiä suhteita, sillä EGF saattaa säädellä Th17-immuniteettia ja siten tyypin 1 diabeteksen kehittymistä.

Avainsanat: Tyypin 1 diabetes, T-solu, D-vitamiini, Th17-immuniteetti, Epidermaalinen kasvutekijä

To Max, Emelie and Benjamin

Contents

1 List of original papers

The doctoral study is based on the following original publications, which are referred to in the text by their Roman numerals (I–IV):

I. Miettinen ME, Reinert L, Kinnunen L, Harjutsalo V, Koskela P, Surcel HM, Lamberg-Allardt C, Tuomilehto J. Serum 25-hydroxyvitamin D level during early pregnancy and type 1 diabetes risk in the offspring. Diabetologia. 2012 May;55(5):1291-4.
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II. Reinert-Hartwall L, Honkanen J, Härkönen T, Ilonen J, Simell O, Peet A, Tillmann V, Lamberg-Allardt C, Virtanen SM, Knip M, Vaarala O; DIABIMMUNE Study Group. No association between vitamin D and β-cell autoimmunity in Finnish and Estonian children. Diabetes Metab Res Rev. 2014
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III. Reinert-Hartwall L*, Honkanen J*, Salo HM, Nieminen JK, Luopajärvi K, Härkönen T, Veijola R, Simell O, Ilonen J, Peet A, Tillmann V, Knip M, Vaarala O; DIABIMMUNE Study Group; DIABIMMUNE Study Group.Th1/Th17 Plasticity Is a Marker of Advanced β Cell Autoimmunity and Impaired Glucose Tolerance in Humans. J Immunol. 2015 Jan 1;194(1):68-75.
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IV. Reinert-Hartwall L, Siljander H, Härkönen T, Vatanen T, Ilonen J, Luopajärvi K, Dorshakova N, Mokurov S, Peet A, Tillmann V, Knip M, Vaarala O, Honkanen J, and the DIABIMMUNE study group. Is gut microbiome independent activation of EGFR pathway a hidden factor related to the risk of immune-mediated diseases in children? Submitted.

*Both authors contributed equally to this study.

Publication (I) was published earlier in the doctoral study: Miettinen ME Vitamin D and Type 1 diabetes. 2017

2 Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
Aab-	Autoantibody-negative
Aab+	Autoantibody-positive
Ab	Antibody
Ag	Antigen
AHR	Aryl hydrocarbon receptor
AITR	Activation-inducible TNFR family receptor
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy/dysplasia
AREG	Amphiregulin
ATP	Adenosine triphosphate
BB	Bio-breeding
BB-DP	Bio-breeding diabetes prone
BB-DR	Bio-breeding diabetes resistant
BCG	Bacillus Calmette–Guérin
BCL-2	B-cell lymphoma 2
BCR	B-cell receptor
BID	BH3 interacting-domain death agonist
BMI	Body mass index
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of differentiation
CIT	Citron kinase

COX-2	Cyclooxygenase-2
C-peptide	Connecting peptide
CSK	Tyrosine-protein kinase
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T-lymphocyte-associated antigen
CV	Coefficient of variation
CYP	Cytochrome
DC	Dendritic cell
DEQAS	Vitamin D external quality assessment scheme
DIPP	Diabetes prediction and prevention
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EMCV	Encephalomyocarditis virus
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FMC	Finnish maternity cohort
FOXP3	Forkhead box P3
GADA	Glutamate decarboxylate autoantibody
GC	Group-specific component
GDM	Gestational diabetes
GITR	Glucocorticoid-induced TNFR-related
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2SO_4	Sulfuric acid
HbA_1c	Glycated hemoglobin A_1c

HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IA-2A	Tyrosine phosphatase-like protein autoantibody
IAA	Insulin autoantibody
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL	Interleukin
IL-17R	IL-17 receptor
IL-22BP	IL-22 binding protein
ILC	Innate lymphoid cell
INS	Insulin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
ITPR	Inositol 1,4,5-trisphosphate receptor
iTreg	Induced Treg
JAK-STAT	Janus kinase-signal transducer and activation of transcription
KIR	Killer-cell immunoglobulin-like receptor
LAG-3	Lymphocyte activation gene 3
Lti	Lymphocyte tissue inducer
Lyp	Lymphoid-specific phosphatase
MARD	Mild age-related diabetes
MHC	Major histocompatibility complex
MOD	Mild obesity-related diabetes
MODY	Maturity-onset diabetes in the young

MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene
NFκB	Nuclear factor κB
NK	Natural killer
NMO	Neuromyelitis optica
NO	Nitric oxide
NOD	Non-obese diabetic
NOS2A	Nitric oxide synthase 2A
nTreg	Natural Treg
PAMP	Pathogen-associated molecular pattern
PASW	Predictive Analytics SoftWare
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Programmed cell death
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PLN	Pancreatic-draining lymph nodes
PRR	Pattern recognition receptors
PTPN	Protein tyrosine phosphatase, non-receptor
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
RIA	Radioimmunoassay
RNASeq	RNA sequencing
ROR	RAR-related orphan receptor
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RU	Relative unit
Runx1	Runt-related transcription factor 1
SFB	Segmented filamentous bacteria

SNP	Single nucleotide polymorphism
SOD2	Superoxide dismutase 2
SPSS	Statistical Package for the Social Sciences
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-related modifier
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T-bet	T-box transcription factor
TCR	T cell receptor
TNF-α	Tumor necrosis factor alpha
TGF	Transforming growth factor
Th	T helper
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TR1	T regulatory type 1
Treg	Regulatory T cell
tTreg	Thymic regulatory T cell
UC	Ulcerative colitis
VDR	Vitamin D receptor
VNTR	Variable number of tandem repeats
ZnT8	Zinc transporter 8

3 Introduction

Type 1 diabetes is one of the most common chronic diseases, often developing during early childhood. Despite decades of research, there is no cure for type 1 diabetes or treatment to stop disease development, and accompanying chronic conditions such as renal, kidney, and eye failures are consequently common in patients with type 1 diabetes. This underlines the importance of research regarding the mechanisms of disease development.

Type 1 diabetes is an immune-mediated disease in which T cells destroy the insulin-producing beta cells in the pancreas. This leads to the loss of sufficient insulin production and, without insulin treatment, death of the patient. The process during which the beta cells are destroyed can continue for a varied amount of time. This period is characterized by the appearance of disease-associated autoantibodies in the blood. The exact molecular mechanisms of this destruction and the factors that lead to it are mostly unknown. The strong genetic susceptibility to type 1 diabetes does not explain the gradual increase in disease incidence, and environmental factors modulating the disease risk therefore need to be studied. Factors thought to be involved in disease development as either triggers or promoters include nutrition, infections, alterations in the microbiota, and toxins. T cell subsets and responses involved in disease development have been investigated to find a way to stop the beta-cell destruction.

This doctoral study aimed at characterizing T cell responses during the preclinical phase of type 1 diabetes. The association between vitamin D and type 1 diabetes, the timing of Th17 immunity during disease development, and the role of mucosal immunity were studied.

4 Review of the literature

The immune system protects us from pathogens, foreign substances, and aberrancies occurring in our own cells. This complex system consists of different organs, cells, and proteins. Along with the nervous system, the immune system is the most complex system that the human body has. The first of the main responsibilities of the body's immune system is to neutralize pathogens such as bacteria, viruses, parasites, or fungi that have entered the body, and to eradicate them. The second task is to recognize and neutralize harmful environmental substances and to tolerate harmless or beneficial commensals, nutritional antigens, and other structures. The third assignment is to fight the body's own harmful cells such as cancer cells. The following chapters of this literature review describe important cells and mechanisms of the immune system, with the emphasis on those important for this study, the pathogenesis of type 1 diabetes and finally the association of environmental factors and immunological aberrancies with type 1 diabetes development.

4.1 The innate immune system

The innate immune system is designated as the first line of defense of an organism against pathogens. Innate immunity consists of multiple different cells that originate from a common myeloid progenitor cell, such as macrophages, granulocytes (neutrophils, eosinophils, basophils), and dendritic cells (DCs). These three cell types are called phagocytes, since they can engulf and kill pathogens and induce inflammation. This attracts other immune cells to the site of infection. Another innate immune cell type is the

natural killer (NK) cell, which is mainly able to recognize and kill abnormal cells such as those infected with viruses and tumor cells.

DCs, macrophages, and neutrophils recognize and distinguish foreign particles with pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and scavenger receptors at the site of infection. They recognize pathogen-associated molecular patterns (PAMPs) such as mannose-rich oligosaccharides, peptidoglycans, lipopolysaccharides, and peptides that many pathogens express. After signal recognition and engulfing of the foreign material, macrophages and DCs migrate into lymph nodes and become active antigen-presenting cells (APCs). In the lymph nodes, the antigen is processed and displayed on the cell surface of the APCs bound to major histocompatibility complex (MHC) molecules. These APCs become activated and produce cytokines and chemokines to recruit more immune cells to the site of infection, and present antigens to T lymphocytes for activation [1]. In humans, MHC molecules are called human leukocyte antigens (HLA), since they were first discovered on leukocytes. MHC class I proteins present antigens to CD8+ cytotoxic T cells, and MHC class II molecules present antigens to CD4+ helper and regulatory T cells. This activates the adaptive immune system [2]. Co-stimulatory molecules such as CD80 and CD86 are expressed on the surface of DCs, and they are necessary for T cell activation through their interaction with the co-stimulatory CD28 molecule on the surface of the T cell [3, 4].

4.2 The adaptive immune system

The response of the adaptive immune system to pathogens is more specific than the response of the innate immune system. The adaptive immune system is highly developed and only present in vertebrates with jaws (gnathostomes) [5]. It generates responses against a wide variety of antigens and

provides long-lasting immunity. The effector cells of the adaptive immune system are lymphocytes, which display a large variety of antigen receptors on their cell surface. These cells respond to antigens by proliferating and differentiating into clonal effector cells [6].

Lymphocytes can be divided into bone marrow-derived B cells responsible for humoral immune responses and thymus-derived T cells responsible for cell-mediated immune responses. Both cell types have their own type of antigen receptor, the B cell receptor (BCR) on B cells and the T cell receptor (TCR) on T cells. T cells can be divided into cytotoxic T cells (CD8+ Tc 4.2.1) and helper cells (CD4+ Th 4.2.2). In order to establish a wide variety of antigen receptors, surface receptors are generated through somatic recombination and hypermutation [7]. BCRs bind to epitopes, which leads to the activation and differentiation of B cells into antibody-secreting plasma cells. Th cells are needed for B cells to differentiate into plasma cells. These then secrete antibodies that bind to target antigens and initiate the neutralization or destruction of the target. In addition to antibody secretion, B cells present antigens to CD4+ Th cells on MHC class II molecules [8]. Recently, Hong et al. reported that B cells were the dominant cell population to present antigens to naïve CD4+ T helper cells upon immunization [9].

T cell development begins with lymphoid progenitors in the bone marrow. These then migrate into the thymus in order to fully mature [10]. Double-positive cells (which express both CD4 and CD8 receptors) are further selected and tested for their T cell receptors (TCRs) to interact with MHC molecules with at least a weak affinity to ensure reactivity [11]. After positive selection, developing naïve T cells are subjected to negative selection in the thymus. During negative selection, self-antigens are presented to the

naïve T cells. Negative selection leads to the deletion of T cell clones carrying TCRs that recognize self-antigens with an excessively high affinity. Mature naïve T cells express either CD4 (Th cells) or CD8 (CTLs) co-receptors and leave the thymus to reside in lymphoid tissues, where antigens are presented to them by activated DCs. Subsequently, activated naïve T cells differentiate into effector and memory T cells. Memory cells are further divided into central memory cells (Tcm) and effector memory cells (Tem). Central memory cells lack immediate effector functions and they home to secondary lymph nodes, where they can be reactivated [12]. Effector memory cells display receptors for homing to inflamed tissues and express immediate effector functions [12]. Memory cells can survive for many years. Different mechanisms are involved in T cell activation, of which the first and second are obligatory: 1) HLA–antigen epitope–TCR interaction, 2) co-stimulation, 3) interaction of cell–cell adhesion molecules, and 4) cytokine secretion by APCs. Conventional T cells cannot bind antigens directly, but the TCR binds antigen epitopes bound to the MHC molecules on the surface of DCs, macrophages, and B cells.

With the help of the innate system, the adaptive immune system is responsible for generating an immunological memory. This leads to rapid recall responses to antigens that have previously been encountered. Both T and B cells can become long-lived memory cells that generate a fast recall response to secondary pathogen challenge [13].

Two important mechanisms preserve immunological homeostasis and the self-tolerance of T cells: central and peripheral tolerance. T cells with an excessively high TCR affinity towards peptides from self-proteins presented on MHC molecules are deleted in the thymus of healthy individuals, and this is referred to as central tolerance [14]. When these autoreactive T cells are not deleted but released from the thymus to the peripheral blood

stream, they can still be controlled through peripheral tolerance mechanisms [15], the most important of which involves regulatory T cells (4.2.2.4). When these mechanisms fail, autoimmunity can occur.

4.2.1 Cytotoxic T cells

Cytotoxic T cells protect against intracellular pathogens residing in the cytosol, such as viruses, some bacteria, and parasites. They kill infected cells before the microbes can proliferate and escape to infect more cells. DCs activate cytotoxic T cells to become effector cells that recognize target cells displaying the same antigen as presented to them by the DC. They express the co-receptor CD8, which recognizes the invariant region of the MHC I protein. Two main mechanisms are responsible for the killing of the target cell: 1) perforin–granzyme-dependent killing and 2) Fas-dependent killing [16-18]. In the first, the T cell releases a pore-forming protein, perforin, to form transmembrane channels in the plasma membrane of the target cell. Serine proteases enter the target cell through the channels and activate apoptosis through the protease granzyme B interacting with a pro-apoptotic protein called Bid, which through its truncated form releases cytochrome c from mitochondria, leading to the caspase cascade and apoptosis. The second mechanism goes through Fas–Fas-ligand activation, where an adapter protein binds to the Fas–Fas-ligand complex and then binds procaspase 8, which in turn becomes activated and leads to the caspase cascade and apoptosis. A third method to enhance the destruction of the target pathogen is the release of cytokines [19]. CD8+ T cells secrete interferon (IFN)-γ and TNF-α, which are able to inhibit viral replication, activate macrophages, and enhance MHC class I expression and apoptosis of the target cell.

4.2.2 T helper cells

Th cells are an important component of the adaptive immune system. They interact with cells of both the innate and adaptive immune system, responding to signals from DCs in particular. As mentioned previously, naïve CD4+ T helper cells differentiate into distinct subsets after ligation of the TCR to the peptide bound to the MHC class II molecule. Co-stimulatory proteins binding to co-stimulatory receptors, cell–cell adhesion formation, and cytokines also direct their differentiation. They display the co-receptor CD4, which binds to the invariant region of the MHC II molecule. A small part of these cells differentiate into long-lasting memory cells. The cytokine environment, the antigen, and the strength of stimulation are factors that affect the different Th cell outcomes (Figure 1). It has previously been thought that different subsets target specific pathogens and secret specific cytokines, but recent results have shown that cells go through different fates during their lifespan rather than commit to a single fate [20].

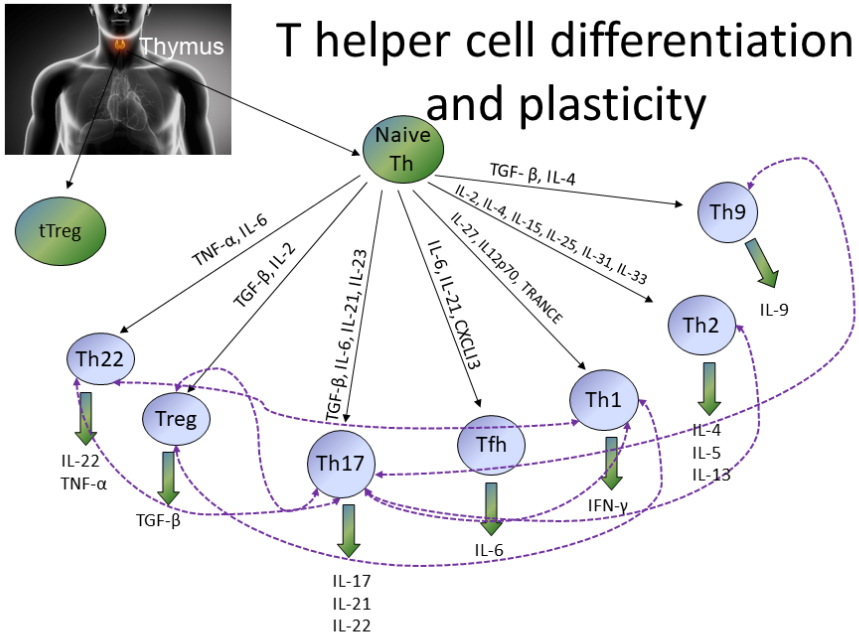

Figure 1. T helper cell differentiation and plasticity. The black arrows describe the cytokines directing the differentiation of different T helper types. The purple lines suggest potential fates for an individual cell. Modified from [21] [22] and [23].

4.2.2.1 Th1 and Th2 cells

The first T helper cells identified were Th1 and Th2 cells [24, 25]. Naïve T cells that are activated by interleukin (IL)-12, producing DCs, differentiate into IL-2 and IFN-γ producing Th1 cells [26]. These cells are activated during infections with intracellular pathogens such as intracellular bacteria and viruses. Factors that favor Th1 differentiation include IFN-γ/signal transducer and activation of transcription-1 (STAT1), IL-2/STAT5, and IL-12/STAT4 signaling, and strong TCR signaling [27]. More specifically, the Janus kinase-STAT (JAK-STAT) pathway plays an important role in signal transduction from the cell membrane to the nucleus of the cell (reviewed in [28]). JAKs are tyrosine kinases associated with membrane-bound cytokine receptors. Receptor activation results in trans-phosphorylation of JAKs,

leading to the recruitment, dimerization, and phosphorylation of STATs. These then migrate to the nucleus and regulate the transcription of their target genes. There are four human JAKs and seven human STATs. Th1 cells need at least TYK2, JAK2, STAT1, and STAT4 for polarization [28]. Th2 cells need JAK1, JAK3, and STAT6 for differentiation. STAT5A/B and STAT3 have also been observed to be involved in Th2 polarization [28]. Th1 cells activate macrophages to destroy infected cells, regulate Ig class switching on B cells, and provide help to CD8+ T cells. Th1 cells are also involved in autoimmunity [27]. The signature cytokine of Th1 cells, IFN-γ, causes increased expression of TLRs, MHCI and MHCII antigen presentation, chemokine secretion, and increased activation and phagocytosis of macrophages [27]. Th2 cells are differentiated under IL-4 signaling and produce IL-4, IL-5, IL-9, IL-10, and IL-13 cytokines. These are important in the fight against multicellular pathogens such as helminths. Overactivation of Th2 cells has been associated with allergies and atopic illnesses. The master transcription factors regulating each fate are T-bet for Th1 cells and GATA3 for Th2 cells.

4.2.2.2 Th17 cells

Th17 cells play an important role in the defense against extracellular bacteria, fungi, and other eukaryotic pathogens, but they are also associated with autoimmune diseases [29]. One of the functions of Th17 cells is to support innate immune responses against extracellular bacteria and fungi in the gut [30] and other mucosal surfaces. The presence of transforming growth factor (TGF)-β, IL-21, IL-6, or IL-1β induces the population of Th17 cells [31, 32]. In addition to IL-17, these cells also produce IL-21, IL-22, IL-25, and IL-26. Th17 cells express the chemokine receptor CCR6, which has only one known ligand, CCL20, and is specific to immune cells originating from the intestine and mesenteric lymph nodes [33] [34]. Th17

cells are commonly found in the lamina propria of the small intestine under homeostatic conditions. The normal microbial flora in the intestine affects the development of Th17 cells. The transcription factor specific for Th17 cells is retinoic acid receptor-related orphan receptor (ROR) γt [35]. Quite recently, it was observed that RORα regulates the same genes as RORγ, but in a slightly weaker way [36]. The involvement of all JAKs, along with STAT3, has been recognized to be important for Th17 polarization [37, 38].

4.2.2.3 Th9 and Th22 cells

Th9 and Th22 cells have been linked to both disease development and the preservation of immune homeostasis, and it remains to be seen what the most important functions of these cells are. Th9 cells are generated in the presence of IL-2, IL-4, TGF-β, and IL-1, while IFN-γ inhibits Th9 differentiation [39, 40]. IL-4 and TGF-β induce expression of the PU.1 transcription factor, leading to the differentiation of Th9 cells [27]. Duhen [41] and Trifari [42] identified the Th22 cell subset. These cells have been described as inflammatory, but they also have a tissue regenerating function. They secrete IL-22 and are differentiated through IL-6 and TNF by inducing the aryl hydrocarbon receptor (AHR) [27]. Both Th9 and Th22 cell subsets need further research on their role in regulating immune responses.

4.2.2.4 Regulatory T cells

Regulatory T cells (Tregs) express CD4, CD25, and the transcription factor FOXP3, and they secrete TGF-β and IL-10. Tregs are usually divided into two groups depending on their origin: thymic-derived (tTregs) and peripherally inducible Tregs (iTregs). tTregs derive from positively selected thymocytes with a relatively high TCR affinity for self-antigens presented on

the MHC II. One of the main functions of Tregs is to maintain self-tolerance. They were first described in mice and subsequently also in humans [43, 44]. Inducible Tregs can be generated from mature T helper cells under the influence of TGF-β and IL-2 [45, 46]. They are usually activated in inflammation, autoimmunity, and cancer. In the mid-1990s, it was noted that in mice, the removal of CD25+CD4+T cells produced a wide range of autoimmune diseases immunologically similar to human diseases such as autoimmune gastritis, thyroiditis, and type 1 diabetes [43]. A third type of regulatory T cells, T regulatory type 1 (Tr1) cells, secrete high levels of IL-10 and express the cell-surface markers CD49b and LAG-3 but lack FOXP3 expression [47].

The transcription factor FOXP3 is needed for the generation and function of Tregs, and patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome suffer from autoimmune conditions such as type 1 diabetes, allergies, and autoimmune skin conditions due to a mutation in the *FOXP3* gene [48-50]. STAT5 binds to the promoter region of *FOXP3* and regulates its transcription [51]. Another marker especially characterizing tTregs is the transcription factor Helios. It has been suggested to be a marker for a thymic origin and for a more active and suppressive phenotype of Tregs [52].

Various mechanisms have been suggested for Treg-mediated suppression. These include cell contact-dependent and humoral factor-mediated mechanisms with the involvement of a wide number of molecules, including cell surface molecules (CTLA-4, CD25 and TIGIT), cytokines (IL-2, IL-10, and TGF-β), and secreted or intracellular molecules (granzyme and cyclic AMP) (reviewed in [53, 54]). Importantly, the expression of FOXP3 in non-Tregs provides them suppressive activity, indicating that FOXP3 is essential in mediating suppression [55].

Beyond functioning in the suppression of inflammation, Tregs are also present in healthy tissue, such as in healthy skeletal muscle, visceral adipose tissue, and the hair follicle stem cell niche [55]. There they are involved in tissue repair and hair regrowth via the production of the growth factors amphiregulin (AREG) and the Notch ligand family member Jagged 1 [56, 57].

4.2.2.5 The plasticity of CD4+ T helper cells

Plasticity of T cells refers to phenotypic switching in response to the local microenvironment of the cells. It has been suggested that effector memory cells are highly plastic, while central memory cells are more stable [58]. Uncontrolled plasticity has been linked to autoimmune diseases such as inflammatory bowel disease, type 1 diabetes, and multiple sclerosis (MS) [59-62] (reviewed in [63]).

The plasticity of Th17 and Treg cells in the context of disease development is explained in more detail in chapters 4.5.3 and 4.5.4.

4.3 The maturation of the immune system

The immune system of a newborn was previously thought to be naïve, but even neonatal T cells proliferate in response to many antigens, including allergens, autoantigens, and parasite antigens [64]. Antigen-specific reactivity has been observed in umbilical cord blood mononuclear cells collected at birth. During fetal life, infections and vaccinations of the mother already affect the immune system of the unborn child. Moreover, Th2 immunity dominates, since Th1 immunity and IFN-γ are highly toxic to the placenta, and Th1 immunity is only upregulated after birth, unless a priming microbial environment is absent during infancy. It has been observed that IFN-γ upregulation in Th cells usually occurs after the first year of life. The principal stimulation of postnatal immune maturation comes from the

microbial environment, especially the commensal microbiota. The maturation of the gut microbiota is an individual process lasting for an indefinite amount of time [65]. Infections in the gastrointestinal tract also affect immune system maturation [64]. Studies have shown that the adult-equivalent levels of CD45RO-positive memory T cells are achieved on average only at the age of 15 years [64].

4.4 Classification of human diabetes

Diabetes in humans has traditionally been divided into four main types: type 1 diabetes, type 2 diabetes (T2D), gestational diabetes (GDM), and other type of diabetes. Approximately 5–10% of individuals with diabetes have type 1 diabetes, which has previously been referred to as insulin-dependent diabetes or juvenile-onset diabetes. Type 1 diabetes can be divided into type A, which is immune-mediated, and type B, which is idiopathic and very rare.

Type 2 diabetes, also referred to as non-insulin-dependent diabetes or adult-onset diabetes, accounts for 90–95% of diabetes patients. It is a disease characterized by insulin resistance or insulin deficiency. Patients are often obese and obesity itself is the most common cause of insulin resistance [66]. Weight reduction and/or medication usually improves insulin responsiveness but never restores it to normal. There is a strong genetic predisposition in T2D, but it is very complex and not fully defined [66].

Other rare diabetes types are often caused by monogenic defects in beta-cell function. Examples of these types are maturity-onset diabetes in the young (MODY), which is characterized by impaired insulin secretion but minimal or no defects in insulin action, and neonatal diabetes, which is diagnosed during the first six months of life and can be permanent or transient.

4.5 Type 1 diabetes

Type 1 diabetes is an autoimmune disease where the insulin-producing beta cells of the Langerhans islets in the pancreas are destroyed. This leads to the loss of blood glucose control and hyperglycemia. If the condition is not treated with exogenous insulin, it can result in ketoacidosis, blindness, renal failure, and cardiovascular disease, and even death [67]. Even with balanced treatment, type 1 diabetes may shorten life by as much as 10 years on average [68]. The criteria for a type 1 diabetes diagnosis are: 1) classic symptoms with plasma glucose concentration ≥ 11.1 mmol/l or 2) fasting plasma glucose ≥ 7.0 mmol/l or 3) two-hour postload glucose ≥ 11.1 mmol/l during an oral glucose tolerance test (OGTT) or 4) HbA1c ≥ 6.5% [69, 70]. Millions of people have been diagnosed with type 1 diabetes worldwide, and young children are especially affected. Approximately 96 000 children under 15 years of age are estimated to develop type 1 diabetes annually [69]. Worldwide, the highest incidence is in Finland, where the incidence peak was observed in 2006 in children aged under 15 years, with 64.9 per 100 000 person years [71]. Type 1 diabetes often begins at an early age, in childhood or adolescence, but it is even possible to be diagnosed with the disease as an elderly person [66]. In 2018, 70% of patients diagnosed in Finland were younger than 20 years old and 15% were over 30 years old [72]. Diagnosed patients older than 50 years are rare, and in Helsinki, Finland, for example, only 1 to 3 are diagnosed per year [72]. Patients with type 1 diabetes are often prone to other autoimmune diseases. The most frequent diseases reported include Hashimoto's thyroiditis and Graves' disease, collectively referred to as autoimmune thyroid diseases (15–30% of type 1 diabetes patients), celiac disease (4–9%), autoimmune gastritis/pernicious anemia (5–10%), Addison's disease (0.5%), and vitiligo (2–10%) [73]. In Finland, hypothyreosis is the most common additional

autoimmune disease, with the disease being three times more common in patients with type 1 diabetes compared to controls (18.1% vs 6.0%) [74]. Mäkimattila et al. observed that hyperthyreosis was present in 2.4% of patients compared with 0.8% of controls, Addison's disease in 0.38% of patients compared with 0.016% of controls, and celiac disease in 4.4% of patients compared with 0.99% of controls [74].

One recent study investigated the incidence rise in children under the age of 15 years in 22 countries, finding an overall increase of 3.4% (95% CI 2.8% to 3.9%) in the incidence rate per annum [75]. The new developing technology of insulin pumps and glucose meters has improved and simplified the efficient treatment of type 1 diabetes patients, but the burden still remains for the rest of the patient's life, and life-long treatment requires high motivation and competence from the patients and their families. Therefore, it is desirable to either try to cure the disease with the help of transplantations or to stop or prevent the destructive process, or even regenerate already lost beta-cell function [76]. One of the most challenging tasks is to arrest the disease process, since the mechanisms leading to it are highly complex and multifactorial. Another factor that makes it difficult to find a solution is the fact that there seems to be high variance in metabolic profiles among patients. Therefore, individualized treatment and profiling should be a goal in the future.

4.5.1 Genetics of type 1 diabetes

It is well known that there is a genetic predisposition to developing type 1 diabetes. Many genetic polymorphisms have been identified to be associated with type 1 diabetes, and certain HLA genotypes seem to be associated with the highest risk of developing the disease [77, 78].

4.5.1.1 Genetic polymorphisms associated with type 1 diabetes

The most important risk genotypes are located in the HLA complex in chromosome six and comprise half of the genetic component [79]. The HLA complex contains at least 128 genes and most of them are involved in immunity [80]. The HLA-DQA1 and HLA-DQB1 genes are the most important determinants of the disease risk, whereas HLA-DR genes have more of a modifying effect. The most common high-risk genotypes are the trans-heterodimers DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*0301-DQB1*0302 (DR3-DQ2/DR4-DQ8) and the most protective genotype is DQA1*0101-DQB1*0602 (DR15(2)-DQ6) [79, 81, 82]. The analysis of DQB1 alleles often provides a good risk estimation when determining the alleles DQB1*02, DQB1*0302 or DQB1*0602 [83].

Numerous non-HLA genes associated with type 1 diabetes risk have been identified, many of them with small odds ratios, but at least four genetic polymorphisms have consistently been observed to be associated with the type 1 diabetes risk in independent studies [84]. These include the insulin (*INS*) gene, the cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), the protein tyrosine phosphatasis pyrosine nonreceptor-type 22 (*PTPN22*) and the IL-2R α-subunit of the IL-2 receptor complex locus (*IL2RA*) [84, 85]. The region around the *INS* gene on chromosome 11p15 was found to be associated with the risk of type 1 diabetes almost 30 years ago [86, 87]. The *CTLA4* gene is located on chromosome 2q33. The role of CTLA4 is thought to be in regulating self-tolerance and hence preventing autoimmunity [85]. CD4+ and CD8+ T cells express CTLA4, which binds B7-1 and B7-2 ligands involved in antigen presentation. The results regarding variants of the *CTLA4* gene are somewhat controversial, but it has been shown that the most strongly associated single nucleotide polymorphism (SNP) is

A6230G in the 3`UTR [88]. *PTPN22* encodes the protein lymphoid-specific phosphatase (Lyp), which is involved in suppressing T-cell activation through TCR signaling by interacting with the C-terminal Src kinase (Csk) [89]. The C1858T variant in the coding region of *PTPN22* is associated with an increased risk of type 1 diabetes, but also of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Graves' disease [85, 90]. The variant leads to a Lyp protein with enhanced binding properties to the Csk kinase, and hence a more efficient suppression of TCR signaling. This can lead to increased survival of autoreactive T cells or modified Treg functions, leading to less effective suppression of autoimmunity [85].

The identified genetic risk factors explain ~60% of the observed genetic predisposition and they have been recorded in several human populations. Other polymorphisms that have been studied but show an inconsistent association with type 1 diabetes are the vitamin D receptor (VDR) polymorphisms, which have been associated with type 1 diabetes in Dutch, German, Bangladeshi Indian and Japanese populations, but not in British or Romanian populations [85]. One rather recent study detected two VDR polymorphisms in Finnish pregnant mothers whose children later developed type 1 diabetes: one in the *VDR* gene (rs4516035) and the other in the group-specific component (*GC*) gene (rs12512631) [91]. Many associations have been revealed in single studies so far, but it is likely that many more will be identified in the future.

4.5.2 Immunological aberrancies in type 1 diabetes

The pathogenetic mechanisms of type 1 diabetes have been studied in animal models such as the bio-breeding (BB) rat since the 1970s and non-obese diabetic (NOD) mice since the 1980s [92, 93]. These animals spontaneously develop autoimmune diabetes and share similarities with the human disease. Both models present with polyuria, glycosuria, weight loss,

and lymphocytic infiltration of the islets (insulitis) [92, 93]. Healthy NOD mice rapidly develop autoimmune diabetes after the transfer of T lymphocytes from diabetic mice [94]. Insulitis caused by Th1 cells secreting IFN-γ leads to beta-cell damage and autoimmune diabetes [95]. Isolated islet-specific CD4+ T cells secrete IFN-γ upon islet antigen stimulation compared with control cells (not islet specific) that secrete IL-10 [96]. In humans, a threshold of >15 CD45+ cells/islet has been established for the definition of insulitis [97]. In human type 1 diabetes, islet cell autoantibodies (ICA) against insulin (IAA), glutamic acid decarboxylase 65 (GADA), islet antigen-2 (IA-2), and zinc transporter 8 (ZnT8) typically appear in the blood before the onset of clinical disease [98-101]. Islet antigen-reactive peripheral blood T cells have also been detected in type 1 diabetes [102]. The presence of autoantibodies probably reflects ongoing islet autoimmunity, and they can be used for disease prediction. The appearance of autoantibodies is considered the first stage of disease development. The presence of a persistent autoantibody, recognizing one antigen, is linked to a slightly increased risk of developing type 1 diabetes. The persistent presence of two or more autoantibodies associates with a high risk of developing the disease, although the time to diagnosis can vary considerably [103]. It has been estimated that the risk of developing type 1 diabetes by the age of 15 years is 0.4% when no autoantibodies are detected. When one autoantibody is present, the risk rises to 14.5%, and with multiple autoantibodies, the risk rises to 69.7% [104]. It has been observed that children with IAA or ZnT8 as their first antibodies are younger than children with GADA or IA-2A as their first antibodies at the time of seroconversion [103]. Children with IA-2A as their first antibody progress more rapidly to clinical disease than children with the other antibodies as their first ones [103]. The second stage of type 1 diabetes development is characterized by dysglycemia along with

the presence of multiple autoantibodies, and the final stage is clinical disease with hyperglycemia. Since the time from seroconversion to clinical disease varies and cannot be fully predicted, it is of great importance to find new biomarkers associated with disease progression to help in disease prediction and possible protection.

The first studies on human islets in patients with type 1 diabetes were published in the 1960s, in which the infiltration of immune cells in inflamed islets was described [105]. Later, it was observed that 40% of the islet-infiltrating mononuclear cells were IFN-γ-secreting cells [106]. Willcox et al. demonstrated that the early phase of insulitis presented with a greater abundance of CD8+ T cells and macrophages and the later phase with CD20+ B cells in large numbers. CD4+ T cells have been observed in all phases of insulitis [107]. It has been observed that there is variation between patients, especially in the number of infiltrating CD20+ B cells [108, 109]. Leete et al. also demonstrated that this difference in the B cell profile could be linked to the age of disease onset, since a CD20lo profile was detected in patients who were diagnosed at an older age (mean 13 years), whereas a CD20hi profile was seen in younger patients (mean age at diagnosis 7 years) [109]. This has resulted in a hypothesis that there are at least two different forms of insulitis in humans and that the CD20hi profile is a more aggressive form [110]. Interestingly, it has been shown that beta cells are still left in the pancreases of some type 1 diabetes patients who have lived with the disease for over 50 years [111]. The possible reasons for this are speculated to be mitosis of beta cells in type 1 diabetes patients and the survival from autoimmune attack of some beta cells by unknown means [112, 113]. It has been recognized that at least four different beta-cell types are present in the islets, and some of them might be more resistant to destruction [114]. The hyperexpression of HLA class I molecules on beta

cells might be a factor separating these subtypes. It has also been observed that hyperexpression of the HLA class I decreases over time [115]. This might be one reason why there are individual forms of disease. Additionally, it has recently been described that α and γ cells isolated from deceased human donors had the ability to differentiate into insulin-producing cells in response to glucose after reprogramming with the transcription factors pancreatic and duodenal homeobox 1 (PDX1) and MAFA [116]. When these cells were transplanted into diabetic mice, the converted human α cells reversed diabetes and continued to produce insulin for a period of six months. Studies in NOD mice have suggested that blocking IFN-γ function with either IFN-γ-specific antibodies [117, 118] or soluble IFN-γ receptors [119] reduces the incidence of spontaneous diabetes and prevents diabetes by transferred splenocytes from diabetic NOD mice [120]. Furthermore, transgenic expression of IFN-γ in beta cells of diabetes-resistant mice induced autoimmunity, resulting in overt diabetes [121]. *In vitro* studies have revealed that IFN-γ is cytotoxic for beta cells. Exposure of human and murine islets to IFN-γ with either IL-1β or TNF-α induced beta-cell death *in vitro*, while IFN-γ alone had no effect on beta-cell death (reviewed in [122]). PBMCs from type 1 diabetes patients produced more IFN-γ upon stimulation compared with healthy controls and patients with Grave's disease [123]. Patients with an activating mutation in an IFN-γ activator (STAT3) display augmented IFN-γ production in T cells and they develop a broad-spectrum autoimmunity that commonly includes type 1 diabetes [124].

The role of IL-17 in pathogenesis was first observed in murine models of type 1 diabetes. Elevated levels of IL-17 transcripts correlated with the progression of insulitis to disease in the pancreas and blood circulation of the NOD mouse [125, 126]. The study of Emamaullee et al. [127] revealed that

Th17 cells were involved in the effector phase of diabetes disease development in the NOD mouse model. They administered IL-25/IL-17E to support Th2 immunity or anti-IL-17 to mice, and when given at the age of 10 weeks, disease was prevented at the age of six months. Furthermore, it has been reported in the NOD mouse model that islet-targeted IL-2 stimulation of FOXP3+Tregs prevents diabetes [128].

In humans, a link between Th17 immunity and type 1 diabetes has been observed in multiple studies [21, 129-133]. Honkanen et al. [21] reported that children with type 1 diabetes had increased IL-17 secretion and expression of IL-17, IL-22, and RORc2, but also FOXP3 transcripts upon T cell activation *in vitro*. Moreover, circulating CD4+ T memory cells in children with type 1 diabetes had the same pattern of IL-17, IL-22, and FOXP3 mRNA upregulation, also suggesting activation of the IL-17 pathway *in vivo*. IL-17 additionally had detrimental effects on human islet cells *in vitro*. It increased the transcription of SOD2 and, together with IL-1β and IFN-γ, the transcription of the inducible isoform of NOS2A and COX-2, which are involved in the inflammatory response in islet cells. Furthermore, it was observed that IL-17 inhibited the mRNA expression of the anti-apoptotic gene *BCL-2* and enhanced the pro-apoptotic effect of IL-1β+IFN-γ in mouse insulinoma cells and primary human islet cells. A study by Marwaha et al. [129] yielded similar results. They observed that children with type 1 diabetes had elevated levels of CD45RA- CD25intFOXP3low cells that were not suppressive, and these cells secreted significantly more IL-17 than other FOXP3+ subsets. Furthermore, they showed that these type 1 diabetes subjects had a higher proportion of both CD4+ and CD8+ T cells that secreted IL-17. It has been reported that isolated human FOXP3+ Tregs secrete IL-17 when they are stimulated with proinflammatory cytokines IL-1β and IL-6, but retain their suppressive function [133]. In a study by Arif

et al. [130], circulating CD4+ T cells from type 1 diabetes patients showed a greater IL-17 response to stimulation with beta-cell-derived antigens compared to control T cells from control subjects. The authors also examined one case where up-regulation of IL-17A, IL-22, and RORC was observed in islets close to diagnosis. Furthermore, they proved that IL-17 contributed to the amount of apoptosis in human islets by increased nitrite release and up-regulation of IL-17RA in beta cells through STAT1 and NFκB. Ferraro et al. investigated the phenotype of Th17 cells and Tregs from pancreatic-draining lymph nodes (PLNs) and circulating blood of type 1 diabetes patients and healthy controls [131]. They found that type 1 diabetes patients had an elevated frequency of Th17 cells in their PLNs but not in their blood compared to controls. Furthermore, Tregs isolated from PLNs of type 1 diabetes patients were observed to be dysfunctional. One further study demonstrated that IL-17A specifically contributes to the development of type 1 diabetes by exacerbating beta-cell apoptosis and enhancing CCL20 secretion in the islets [132].

4.5.3 The plasticity of Th17 cells

Accumulating evidence suggests that T-cell plasticity, phenotypic switching, and the polyfunctionality of T helper cell subsets is a phenomenon associated with tissue destruction in autoimmune and inflammatory diseases. Originally, these mechanisms probably evolved to enhance the capacity of our immune system to deal with changing challenges caused by diverse pathogens and environmental antigens. However, dysregulation of this system can enhance the risk of autoimmunity.

Th17 cells show substantial phenotypic plasticity. In addition to IL-17, they are also able to secrete other effector cytokines. Animal studies suggest that the plasticity of Th17 cells and the development of IL-17/IFN-γ or IL-

17/GM-CSF co-producers, in particular, is associated with the pathogenicity of Th17 cells, for instance in multiple sclerosis (MS) [63]. Animal studies have demonstrated that Th17 cells from BDC2.5 mice (NOD mice expressing the BDC2.5 transgenic TCR) induce autoimmune diabetes in healthy recipients after their phenotypic shift to Th1 cells *in vivo*. The expression of IFN-γ was upregulated and that of IL-17 downregulated in these cells, and they infiltrated the islets and transferred diabetes [134, 135]. When the IFN-γ response was neutralized with antibodies, diabetes was inhibited, suggesting that the generation of a Th1 response from Th17 cells is important for disease development. The conversion of Th17 cells into double-positive Th1/Th17 cells has also been observed in humans with juvenile arthritis and Crohn's disease [62, 136]. Moreover, it has been reported that Th17 cells can convert into IL-4-secreting Th2/Th17 cells (rev. [63]).

It has been suggested that the IL-23 signaling pathway plays an important role in Th17 plasticity by decreasing FOXP3 expression and increasing IFN-γ secretion. In addition, the cytokine microenvironment is an important factor affecting this. Naïve T cells that are differentiated with IL-1β, IL-6, and IL-23 differentiate into Th17 cells that are highly pathogenic, causing neurological symptoms in experimental autoimmune encephalomyelitis (EAE), the most commonly used experimental model for MS [137]. Psoriasis has been successfully treated with the blockade of the IL-12/IL-23 p40 subunit, which leads to the decreased production of proinflammatory cytokines and the p19 subunit of IL-23 [138]. It has also been reported that Th17 cells specific to different antigens have distinct phenotypes in humans. *Staphylococcus aureus*-specific Th17 cells produce IL-10, whereas *Candida albicans*-specific Th17 cells produce IFN-γ [139]. A

RORγt inverse agonist (TMP778) blocked human Th17 and Tc17 cell differentiation and also modulated IL-17A production and inflammatory Th17-signature gene expression (*IL17A*, *IL17F*, *IL22*, *IL26*, *CCR6*, and *IL23*) in mature human Th17 effector/memory T cells [140]. Even though the number of double-positive IL-17+/IFN-γ+ cells was not altered upon administration of a ROR inverse agonist, the expression of both IL-17 and IFN-γ was inhibited at the mRNA level in NOD mice [141]. This also prevented hyperglycemia and suppressed insulitis.

In 2015, it was shown that Th17 cells can have a dual role in the autoimmunity process in NOD mice. Bellemore et al. observed that CD4+ T cells from BDC2.5 mice could be differentiated into IL-22-producing, disease-initiating Th17 cells driven by IL-23 and IL-6, or through stimulation with TGF-β + IL-6 into non-diabetogenic regulatory Th17 (Treg17) cells expressing high levels of AHR, producing IL-10 but reduced levels of IL-22 [142].

IFN-γ+IL-17+ double-positive T cells have been observed in the colon lamina propria of IBD patients (reviewed in [60]). Th17 cells that line the gut mucosa do not normally induce inflammation, but have been shown to be necessary for the maintenance of normal barrier function of the gut. Commensal bacteria in the gut play a critical role in the differentiation of Th17 cells in the lamina propria. One recent study demonstrated that segmented filamentous bacteria (SFB) induced Th17 cells with a dual TCR expression against both SFB epitopes and autoantigens from the lung, and this led to autoimmunity [143].

4.5.4 The plasticity of Treg cells

The role of Treg plasticity in disease has been investigated, but the results remain inconsistent. In murine models of type 1 diabetes, converted Tregs have been recognized to be pathogenic [144].

FOXP3+IL-17+ double-positive T cells have been observed in the colon lamina propria of IBD patients (reviewed in [60]). Double-positive FOXP3+IL-17+ T cells were observed in higher frequencies in the blood of patients with UC and Crohn's disease compared to healthy controls [59]. Putnam et al. investigated the number and suppressive ability of CD25high Tregs in type 1 diabetes patients and healthy controls and did not find any differences between them [145]. McClymont et al. also studied the plasticity of Tregs in type 1 diabetes patients [61]. They found that the frequency of Tregs was similar in the peripheral blood of type 1 diabetes patients and healthy controls, but the number of double-positive FOXP3+IFN-γ+ Treg cells was higher in patients. A study investigating the role of Treg plasticity in MS, on the other hand, observed a higher frequency of IFN-γ-secreting FOXP3+ Tregs in MS patients compared with healthy controls [146]. These cells also displayed a reduced suppressive capacity when cultured *in vitro*.

4.5.5 The destruction of beta cells in type 1 diabetes

The prediabetic period, during which beta-cell destruction occurs, can vary from a few months to more than a decade (reviewed in [147]). Currently, there is no golden standard for direct assessment of the death of human beta cells. The best method to indirectly assess beta-cell destruction or the functional beta-cell mass is to measure C-peptide, the peptide that connects proinsulin's A- and B-chain, which is co-secreted in a one-to-one molar ratio with insulin but is not first-pass cleared from the circulation by the liver

[148]. The C-peptide levels appear to be lower and hence beta-cell destruction is more severe in recent-onset patients younger than 7 years [110]. It is challenging to investigate the molecular mechanisms of beta-cell destruction in humans, since the pancreas is a retroperitoneal organ, only accessible for studying after the death of the patient, and identifying such cases in the prediabetic phase of the disease is difficult. It has been estimated that only around 150 pancreases at disease onset or shortly after were examined between the years 1902–2010 [149]. This has led to a few biobanks collecting pancreatic samples from diabetic patients, but the number of recent-onset samples nevertheless remains small [110]. Therefore, many results regarding molecular mechanisms come from animal models and other human samples such as blood cells. It is thought that apoptosis is the main pathway of human beta-cell death [107].

Different extracellular signals, ATP levels, phosphorylation cascades, and the expression of pro- and anti-apoptotic genes regulate apoptosis. The process that leads to beta-cell death, the caspase pathway, involves molecules such as the Fas ligand (FasL), perforin, TNF-α, IL-1, and nitric oxide (NO) [150]. Interaction with other effector molecules such as IFN-γ and IL-17 enhances the progression of apoptosis [21, 130]. Studies in animal models have revealed that the first cells infiltrating islets are macrophages and dendritic cells that present beta-cell antigens on their MHC class II molecules to naïve CD4+ T cells in PLNs [151]. This favors Th1 differentiation of the T cell, and these cells further enhance the destructive process by secreting IL-2 and IFN-γ, which leads to the secretion of IL-1β and TNF-α and release of free radicals, such as NO, from APCs and beta cells [151]. CD4+ T cells can also activate B cells to produce antibodies against beta-cell an-

tigens. The cytokine milieu recruits CD8+ T cells to the islets and stimulates the beta cells to secrete IL-15, further enhancing the attraction of immune cells [152].

CD8+ T cells can directly cause beta-cell death by secreting perforin and granzyme to the extracellular space, which causes membrane disruption in the target cell. This occurs when the beta cell presents self-antigens on MHC class I molecules that are recognized by the TCR of the CD8+ T cells [151]. The Fas/Fas L interaction between the beta cell and the CD8+ T cell also contributes to the apoptosis process by activating caspase-8 and caspase-3, leading to cell death.

IL-17 alone or in combination with IL-1β/IFN-γ up-regulates SOD2, COX-2, and NOS2A expression in human islets. IL-17 causes inflammation and increases apoptosis. IL-17 alone also downregulates BCL-2 expression in the islet cells [21].

Cytokine combinations such as IL-1β/IFN-γ and TNF-α/IFN-γ cause STAT1 and NF-kB apoptosis pathway activation and up-regulation of IL-17 receptor (IL17R) expression in human islets, rat beta cells, and INS-1E cells [130]. IL-17 signaling can thus promote inflammation in the cells and enhance the apoptosis process.

4.6 Environmental risk factors for type 1 diabetes

The reason why environmental factors have been implicated in disease development in type 1 diabetes is the fact that disease concordance for monozygotic twins is only about 50%, and that incidence varies greatly in environmentally different populations with a similar HLA genotype background [153, 154]. What furthermore emphasizes the importance of environmental factors is the fact that the incidence of type 1 diabetes is rising in individuals with moderate or low-risk HLA genotypes [69]. It has also

been observed in immigrant populations that the environment predisposes to the risk of developing type 1 diabetes [155-157]. The rate of the growth in type 1 diabetes incidence observed is also too rapid to be accounted for by genetic factors. Moreover, it has to be noted that the incidence drastically differs between regions with genetically similar populations but different childhood environments, such as Espoo in Finland, Tarto in Estonia, and Petrozavodsk in Russia, where the respective incidence rates have been estimated to be 57.6/100 000, 17.1/100 000, and 12.1/100 000 in children under the age of 15 years [158]. It is thought that environmental triggers act during the fetal period and/or childhood either alone or by interacting with genetic factors. This interaction determines whether a genetically-susceptible individual eventually develops the disease.

4.6.1 Infections

Due to the higher incidence rate of type 1 diabetes during fall and winter, infections have already been suggested to be a risk factor for type 1 diabetes since the 1920s. Moreover, quite recently, it was reported again that most diagnoses are made during the period from November to February [159]. A few years ago, it was reported that during winter, a more pro-inflammatory transcriptomic profile of genes is prevalent [160]. The viral infections most strongly associated with the risk of type 1 diabetes development are infections caused by single-stranded RNA enteroviruses of the Picornaviridae family [67]. There are more than 60 serotypes of enteroviruses, and the most widely known are the polioviruses [161]. Enterovirus infections are common among children and adolescents, and often remain subclinical or cause only mild respiratory symptoms. The first studies considering enterovirus infections as a risk factor for type 1 diabetes were conducted as much as thirty years ago [162]. Antibodies against coxsackievirus B serotypes

were detected in newly diagnosed patients with type 1 diabetes compared with controls [163]. One meta-analysis showed that enteroviral infections were associated with type 1 diabetes-associated autoimmunity and clinical type 1 diabetes at odds ratios of 3.7 and 9.8 [164]. Increased T cell responses to enteroviral antigens have been reported in patients with signs of beta-cell autoimmunity and patients with type 1 diabetes [161]. Children positive for multiple beta-cell autoantibodies have been observed to have more enterovirus infections than control children, suggesting the involvement of these infections in the preclinical phase of type 1 diabetes [165]. Children with type 1 diabetes-associated autoimmunity had more and earlier infections than control children [166]. Coxsackie enterovirus infection has been more commonly detected in beta cells of type 1 diabetes patients compared to controls [167]. Enteroviral RNA is also more often found in diabetic patients, suggesting a longer persistence of the infections in patients compared to healthy controls or celiac disease patients [168]. On the other hand, there have also been studies with conflicting results from Italy and Germany [67]. Enteroviral infections *in utero* have also been suggested to increase the risk of type 1 diabetes development, although a recent study concluded that it is not a major risk factor for disease development [169-171]. Respiratory infections have been linked to autoantibody seroconversion in a German study and encephalomyocarditis virus (EMCV)-induced autoimmune diabetes in specific mouse strains and milder disease in other animal models [67]. The exact mechanism by which viruses could induce type 1 diabetes is still unknown and needs further investigation.

4.6.2 Duration of breastfeeding

The effect of breastfeeding on type 1 diabetes development has been studied and it has been suggested that a longer period of breastfeeding may be

protective [172, 173]. A short exclusive breastfeeding time has been associated with autoantibody development in children with the HLA-DQ*0302 risk allele [172]. A recent study also found that children with type 1 diabetes were breastfed for a shorter period and introduced to cow's milk earlier than their siblings [174]. Since many studies have been based on questionnaires, there is a risk of false results, and more research regarding the protective effect of breastfeeding is needed.

4.6.3 Nutrition

The role of nutrition at an early age has been investigated regarding type 1 diabetes development. Cow's milk proteins and cereals have been examined as risk factors. Studies from NOD mice have revealed that highly hydrolyzed casein formula lowered the incidence of diabetes [175, 176]. The use of highly hydrolyzed casein formula reduced the incidence of type 1 diabetes-associated antibodies in young children with a genetic predisposition by more than 50% [175-177]. Moreover, the removal of bovine insulin from cow's milk formula also significantly lowered the incidence of beta-cell autoimmunity in young children with a genetic predisposition compared to children who received a whey-based formula [178]. However, these results could not be replicated in a large cohort study, in which no difference in the risk of developing type 1 diabetes was detected in children who received a hydrolyzed formula compared with a conventional formula [179].

Since celiac disease is more common in type 1 diabetes patients than in healthy subjects, and around 10% of patients suffer from it, the role of gluten has been investigated and controversial results have been found [67]. Results from NOD mice indicate a clear prevention of autoimmune diabetes with a gluten-free diet [180]. In addition, when a soy-based diet was

compared with a wheat-based diet, it was seen that a Th1-type response was induced by the wheat-based diet [181]. A gluten- and cereal-free diet influences the gut microbiota and hence contributes to immune system maturation. In an interesting case report, a six-year-old boy diagnosed with type 1 diabetes had lived for three years without daily insulin shots by changing to a gluten-free diet [182]. Recently, it was found that later introduction of gluten to the diet compared with earlier introduction was associated with a higher risk of autoimmunity (<4 months compared with 4–9 months and >9 months) [183].

4.6.4 Vitamin D

Vitamin D and type 1 diabetes have been linked, because there is geographical variation in the prevalence of type 1 diabetes and vitamin D synthesis in the skin, with a gradient from the north to the south. In 2003, vitamin D fortification of milk and spreads was started in Finland, and this has been suggested as a potential explanation for the plateauing of the incidence rate of type 1 diabetes [71]. *In vitro*, vitamin D has been shown to protect rat pancreatic islets from IL-1β-induced destruction [184]. Vitamin D has been linked to enhanced FOXP3 expression in Tregs and cholecalciferol supplementation has been shown to improve the Treg suppressive capacity in patients with recent-onset type 1 diabetes [185, 186]. This suggests that vitamin D could be a beneficial immunomodulatory agent in combination therapy strategies for type 1 diabetes. The vitamin D status affects insulin secretion, as vitamin D increases intracellular calcium, which in turn decreases insulin secretion [187]. Infections that can be associated with the autoimmune process of beta-cell destruction can be controlled by vitamin D, and it may directly reduce the autoimmune process of beta-cell destruc-

tion [188]. Maternal vitamin D deficiency and its effect on fetal development have been investigated in different studies. It has been observed that serum 25-hydroxyvitamin D (25(OH)D) concentrations are similar in the mother and the newborn [189]. Pregnancy is characterized by a Th2-dominant T helper profile, and this shifts towards Th1 after delivery [190]. This, together with a drop in serum 25(OH)D after birth, could affect the autoimmune process in newborns genetically at risk of developing type 1 diabetes [191]. It seems that an increased maternal vitamin D intake via food or cod liver oil products has a risk-lowering effect on type 1 diabetes development in the offspring, whereas vitamin D intake through supplements or multivitamins has no effect on type 1 diabetes development. This, on the other hand, means that the effect can also be due to other components and not vitamin D alone. The intake of vitamin D in early life has been associated with a lower risk of developing type 1 diabetes in a Finnish study [192]. On the other hand, it must be noted that children in Russian Karelia have a similar genetic susceptibility and circulating vitamin D concentrations as Finnish children and still have a six times lower risk of developing type 1 diabetes [153, 193].

4.6.5 The “hygiene hypothesis” or “biodiversity hypothesis” and gut microbiota in autoimmune disease

The “hygiene hypothesis” postulates that autoimmune diseases are more common in countries with higher hygiene standards. This fits with the higher incidence of type 1 diabetes in countries having stricter hygiene [194]. People in these countries are less exposed to infectious agents, symbiotic microorganisms, environmental microbes, and parasites, and this leads to an altered T cell balance, which might result in the development of autoimmune diseases [67]. The more recent “biodiversity hypothesis”

states in more detail that our contact with natural environments enriches our microbiome, enhances the immunological balance, and protects us from allergy and inflammatory diseases [195]. Two layers of biodiversity protect us, the microbiota of the outer layer (soil, natural waters, plants, animals) and the inner layer (gut, skin, airways). Changes in the diet and antibiotic use have also contributed to the loss of biodiversity. Evidence from animal models supports the hypothesis. For example, NOD mice from cleaner colonies have a higher incidence of autoimmune diabetes [196]. The myeloid differentiation primary response gene (*MyD88*) knockout NOD mouse is a further example of how the microbiota affects diabetes development. MyD88 is an innate immunity-signaling molecule involved in toll-like receptor (TLR) signaling. Deletion of the *MyD88* gene leads to alteration of the gut microbiota composition and the prevention of autoimmune diabetes in these mice under normal housing conditions [197]. Infants born naturally gain their gut microbiota from the mother, whereas infants born through cesarean section gain their gut microbiota from the hospital environment. Birth by cesarean section has been associated with the risk in developing type 1 diabetes [198]. Korpela et al. recognized that prematurely (PT) born infants (<35 weeks of gestation) had a less diverse gut microbiota than full-term (FT) born infants [199]. Fouhy et al. observed that the impact of gestational age at birth persisted for many years. They found that PT children had a similar gut microbiota diversity at 4 years to FT children at 2 years [65]. One of the first studies to show the importance of the gut microbiome in human type 1 diabetes patients revealed that children with signs of beta-cell autoimmunity developed a less diverse and less stable microbiome than their controls at toddler age [200]. A study by de Goffau et al. demonstrated a low abundance of lactate-producing and butyrate-producing gut bacterial species in children with signs of beta-cell

autoimmunity [201]. They also found an increased abundance of the *Bacteroides* genus in these children, consistent with previous results.

Other findings from human studies suggest that gut microbiota composition affects the development of type 1 diabetes. Data from genetically predisposed infants during the time between positivity for autoantibodies and clinical type 1 diabetes suggests that there is a drop in diversity during this time and a peak in the presence of inflammatory organisms, gene expression, and metabolites [202]. Probiotics are recommended for infants in some maternity and child health clinics in Finland from the age of two weeks along with the recommended vitamin D supplement. This recommendation may vary between communes and is not a general guideline. In a recent study that included samples from Finland, it was shown that early exposure (0–27 days after birth) to probiotics reduced the risk of autoimmunity in children with high-risk genotypes for type 1 diabetes compared to later probiotic supplementation, or to no supplementation [203].

The decrease in helminth infections in developed countries has also been speculated to be linked with the growing incidence of type 1 diabetes [67]. Different helminth parasites inhibited or slowed down autoimmune diabetes development in NOD mice [204-206]. No human studies regarding helminthes as vaccines in type 1 diabetes prevention have been reported yet, but promising results have been seen in pilot clinical trials involving patients with IBD and MS [207, 208]. The side-effect of helminths is that they cause chronic IgE-mediated basophil and mast cell activation, which can be avoided by using antibodies that bind to the IgE receptor on basophils and mast cells [209].

4.6.6 Gut inflammation, immunity and type 1 diabetes

The role of gut leakiness, or increased intestinal permeability, has been studied in the context of type 1 diabetes since the late 1990s. Increased

permeability was observed in the stomach, small intestine, and colon of BB rats [210]. Later, it was shown that high-risk diabetes-prone rats (BB-DP) had increased intestinal permeability compared to low-risk diabetes-resistant rats (BB-DR) during the prediabetic phase [211]. Alterations in the tight junction protein claudin were also seen. In humans, the protein zonulin, which regulates intestinal permeability by modulating intracellular tight junctions of the gut epithelium, appears to be upregulated in patients with type 1 diabetes [212]. It has been suggested that the leakiness of the gut influences the immune response against self- and non-self antigens, and this might contribute to disease development. Lee et al. investigated gut barrier disruption and the rate of insulitis in NOD mice upon challenge with different strains of the bacterium *Citrobacter rodentium* [213]. They demonstrated that mice infected with the wild-type strain developed insulitis faster than mice infected with a mutant strain that did not disrupt the gut barrier. They also found that infected mice had increased numbers of pancreatic-draining lymph node T cells compared with non-infected mice. Interestingly, it has been observed that IL-22 is important in protective immunity against *Citrobacter rodentium*. Zheng et al. noticed that IL-22 knockout mice died from *Citrobacter rodentium* infection and histological samples revealed increased mucosal hyperplasia and submucosal inflammation compared to wild-type mice [214]. One study presented results from *in vitro* experiments in which the stimulation of keratinocytes with the supernatant of Th22 cells, or recombinant IL-22 and TNF-α, successfully inhibited *Candida albicans* infections and preserved epithelial health [215]. IL-22 is important in host defense and the preservation of mucosal integrity, but also in the inhibition of cytokine-induced beta-cell damage and induction of beta-cell regeneration in the NOD mouse [216-218]. The absence of IL-22 has led to systemic inflammation and delocalization of

the commensal bacterium *Alcaligenes* in IL-22 deficient mice. *Alcaligenes xylosoxidans*-specific IgG concentrations have been seen in patients with Crohn's disease [219].

Another factor related to the preservation and repair of the intestinal mucosa is epidermal growth factor (EGF) [220]. In the gastrointestinal tract, EGF participates in the maturation and maintenance of the intestinal epithelium [221, 222]. During intestinal development, EGF has a significant role in establishing a selective intestinal barrier function (reviewed in [223]). Seven ligands have been reported to bind to and activate the EGF receptor (EGFR) (reviewed in [224]). These ligands belong to a family of type II cytokines (so-called EGF-like family) that includes EGF, transforming growth factor-alpha (TGF-α), heparin-binding EGF-like growth factor (HBEGF), betacellulin (BTC), AREG, epiregulin (EREG), and epigen (EPGN). Many studies in mice have suggested a link between EGF and diabetes [213, 225-227]. These have reported that EGF deficiency is associated with diabetes, and that EGF induces beta-cell replication and insulin release, and together with ciliary neurotrophic factor (CNTF) restores insulin production in damaged mouse beta cells. Recently, it was observed that in newly diagnosed T2D patients, the concentration of the soluble receptor of EGF (sEGFR) was elevated and correlated positively with blood glucose levels but negatively with C-peptide and insulin levels [228].

5 Aims of the study

"A current goal is to identify modifiable environmental factors or therapeutics that mimic the effects of protective factors or alleles." – John Todd [229]

The aims of this study were to:

I. Investigate the role of vitamin D during the fetal period for type 1 diabetes. We compared serum 25(OH)D levels during early pregnancy in mothers of children who developed type 1 diabetes with those in control mothers of non-diabetic healthy children of the same age.

II. Investigate whether plasma 25(OH)D concentrations associate with the development of beta-cell-associated autoantibodies and the transcriptional activity of FOXP3 or the vitamin D3 convertase gene (CYP27B1) in CD4+ memory T cells.

III. Study temporal associations between the disease pathogenesis of type 1 diabetes and upregulation of Th17 immunity and T cell plasticity.

IV. Investigate markers of mucosal immunity in young children from Russia, Finland, and Estonia.

6 Subjects and Methods

6.1 Subjects

6.1.1 Subjects in study I

Serum samples have been collected from all pregnant women in Finland since 1983 (the Finnish Maternity Cohort, FMC). The FMC stores first-trimester serum samples required for screening of infectious diseases from nearly all (98%) mothers during every pregnancy. Samples are stored at -20 °C for future use.

For this study, 498 case mothers were selected using the Finnish Pediatric Diabetes Register. For each case mother, a control mother was selected according to the withdrawal date of the serum sample. This resulted in 343 case–control pairs. Samples from pregnancies of healthy siblings were collected from 137 case mothers.

The mean storage time of the serum samples before vitamin D analysis was 14.7 years (range 10–17 years). Samples were collected between the years 1993–2000. The mean age at type 1 diabetes diagnosis was 3.4 years (range 0–7 years). Written informed consent was obtained from all mothers and the ethics committee of the Hospital District of Helsinki and Uusimaa approved the study.

6.1.2 Subjects in study II

Children participating in the Finnish and Estonian segment of the DIABIMMUNE study and the Finnish Type 1 Diabetes and Prediction (DIPP) study were screened for HLA-conferred susceptibility to type 1 diabetes and for seropositivity to beta-cell autoantibodies. Fifty-four (47%) children

carried risk-associated HLA-DR/DQ genotypes (nine with a strongly increased, 34 with a moderately increased, and nine with a slightly increased risk). Blood samples were collected between October 2010 and August 2011. Cell separations were performed the day after blood collection. Subject characteristics are presented in Table 1. The local ethics committees approved the study and written informed consent was obtained from the parents of all participants.

Table 1. Subjects in study II

Study	Number of participants	Average age ± SD in years	Female/Male	Autoantibody-positive
DIABIMMUNE (Finnish segment)	43	3.9 ± 0.6	31/12	12
DIABIMMUNE (Estonian segment)	32	4.4 ± 0.4	24/8	6
DIPP	40	8.0 ± 3.0	18/22	18

6.1.3 Subjects in study III

This study included samples from 159 children. Of these, 28 were Finnish children with type 1 diabetes from Helsinki Children's Hospital, 90 children (57 Finnish and 33 Estonian) participated in the DIABIMMUNE study, and 41 participated in the DIPP study. The characteristics of the different study groups are presented in Tables 2 and 3. The categories based on OGTT testing were: 1) 2-hour post-load plasma glucose <7.8 mmol/l = normal glucose tolerance, 2) 2-hour post-load plasma glucose 7.8 to <11.1

mmol/l = IGT, and 3) 2-hour post-load plasma glucose ≥ 11.1 mmol/l = provisional diagnosis of type 1 diabetes, which has to be confirmed with repeated testing [69].

Table 2. Subjects for the study on the up-regulation of Th17 immunity

	Number	Average age ± SD in years	Female/ Male
Type 1 diabetes [T1D]	15	8.3 ± 5.5	6/9
Early beta-cell autoimmunity [AAb+]	29	5.7 ± 3.1	16/13
Advanced beta-cell autoimmunity and impaired glucose tolerance [IGT]	9	7.7 ± 4.3	2/7
Negative for beta-cell autoimmunity [Aab-]	80	4.8 ± 2.3	50/30

Table 3. Subjects for the study on the plasticity of Th17 cells

	Number	Average age ± SD in years	Female/ Male
Type 1 diabetes [T1D]	13	10.2 ± 5.1	6/7
Early beta-cell autoimmunity [Aab+]	5	5.8 ± 3.5	1/4
Advanced beta-cell autoimmunity and impaired glucose tolerance [IGT]	6	9.2 ± 4.5	0/6
Negative for beta-cell autoimmunity [Aab-]	8	5.5 ± 2.3	5/3

Blood samples were analyzed the day after collection. The parents of the study subjects provided their written informed consent before sample collection. The local ethics committees approved the study.

6.1.4 Subjects in study IV

In this study, we collected serum samples from 3-, 6-, 12-, 18-, 24-, and 36-month-old children from Russia (60 children, n = 144), Estonia (46 children, n = 128), and Finland (45 children, n = 126) from the birth cohort of the DIABIMMUNE study. Heparinized peripheral blood samples were collected from the DIABIMMUNE young children's cohort from 48 Finnish and 37 Estonian children. Children in the birth cohort had HLA-conferred susceptibility to type 1 diabetes, whereas children studied in the young children's cohort represented the general population in Finland and Estonia. All studied children were negative for type 1 diabetes-associated autoantibodies. The parents of the study subjects provided their written informed consent before sample collection. The local ethics committees approved the study.

6.2 Methods

Table 4. Laboratory methods used in this doctoral study.

Cell isolations and cultures	**Method**	**Publication**
PBMC separation from peripheral blood	Ficoll isogradient centrifugation	II, III, IV
Enrichment of CD4+ T memory cells	Magnetic depletion	II, IV
Purification of Th17 cells	Secretion assay and sorting with FACSAria II	III

αCD3+αCD28 stimulation of PBMCs for 40 or 72h	Cell culturing	III
Flow cytometry and cytokine analyses		
CD3, CD4, CD45RO, CD45RA	Extracellular staining	II, III, IV
IL-17, IFN-γ	Secretion assay and sorting with FACSAria II	III
High-sensitivity T cell assay	Luminex	IV
38-plex Immunology Multiplex assay	Luminex	IV
Th17 multiplex	Luminex	IV
EGF, TGF-α, sCD40L	Luminex	IV
Serum and plasma 25-hydroxyvitamin D and 1,25-dihydroksyvitamin D measurements		
Serum levels of 25(OH)D	EIA	I
Plasma levels of 25(OH)D	EIA	II
Plasma levels of $1,25(OH)_2D$	Delipidation and EIA	II
Plasma amphiregulin measurements		
Plasma amphiregulin	EIA	IV
RNA isolation		

Total RNA isolation from PBMCs, CD4+ T memory cells and Th17 cells	Filtration technique	II, III, IV
Gene expression analyses		
FOXP3, CYP27B1, 18s1	RT-PCR and TaqMan	II
IL-17A, IL-17F, IL-22, IL-9, IFN-γ, RORc, AHR, FOXP3, 18s	RT-PCR and TaqMan	III
IL-22, IL-17A, IFN-γ, FOXP3, 18s	RT-PCR and TaqMan	IV

6.2.1 Cell isolations and stimulations

Plasma samples were collected before Ficoll (GE Healthcare, Uppsala, Sweden) isogradient centrifugation from fresh heparinized blood samples and stored at -70 °C until analyzed. Peripheral blood mononuclear cells (PBMCs) were washed three times with phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) and re-suspended in serum-free X-VIVO 15 culture medium (Lonza). CD4+ memory T cells in studies **II** and **IV** were separated from freshly isolated PBMCs by one-step magnetic depletion of naïve CD4+, CD45RA+, CD8+, γ/δ, B, natural killer (NK), dendritic, and erythroid cells, monocytes and granulocytes (Miltenyi Biotec, Gladbach, Germany). Cells were lysed in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) for reverse transcription quantitative polymerase chain reaction (RT-qPCR) and stored at -70 °C until analyzed. According to flow cytometry analysis, the purity of the CD4+CD45RO+memory cell preparations was typically greater than 93%.

In study **III**, Th17 cells were sorted after 72 h PBMC stimulation. To examine Th17 immunity, 2 x 10^5 PBMCs/well were cultured in 200 µl volume, in triplicate for 40 h and 72 h, using 96-well round-bottom plates (Thermo Scientific, Waltham, MA). Cells were stimulated with plate-bound anti-CD3 (5 µg/ml in PBS, BD Pharmingen, San Diego, CA) and soluble anti-CD28 (1 µg/ml, BD Pharmingen). Stimulated cells were either collected and lyzed in Qiagen RLT lysis buffer (40 h; Qiagen, Hilden, Germany) for RT-qPCR or collected and labelled with the IL-17 secretion assay kit (72 h; Miltenyi Biotec, Gladbach, Germany) followed by optional staining with anti-CD4-allophycocyanin. Th17 cells were isolated using FACSAria II (BD Biosciences, San Jose, CA, USA). Sorted CD4+ $Th17^{high}$ and CD4+ $Th17^{int}$ cells were lysed in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) for RT-qPCR.

6.2.2 Flow cytometry and cytokine analyses

The purity of CD4+ memory T cells in study **II** and **IV** was regularly assessed during the study by staining the population of CD4+CD45RO+ cells with the following surface antigens: CD4-PE, CD3-PerCP, CD45RO-APC, and CD45RA-FITC (BD Biosciences, San Jose, CA, USA). The isotype controls used were IgG_1-FITC, IgG_1-APC, IgG_1-PerCP, and IgG_1-PE (BD Biosciences, San Jose, CA, USA).

To verify our results for IFN-γ expression at the protein level, the analysis of IL-17 and IFN-γ secretion in study **III** was performed with samples from two autoantibody-negative children, three children with advanced beta-cell autoimmunity, and two children with type 1 diabetes. Frozen PBMCs were thawed and allowed to recover in X-VIVO 15 culture medium (Lonza, Verviers, Belgium) at 37 °C for four hours. The cells were subsequently stimulated for 72 h as freshly isolated PBMCs. After stimulation, the cells

were labelled with IL-17-allophycocyanin and IFN-γ-PE secretion assay kits (Miltenyi Biotec, Gladbach, Germany), CD4-FITC, and 7-aminoactinomycin D (7-AAD). Viable CD4+ T cells were analyzed and single- or double-positive IL-17 and IFN-γ cells were sorted and further analyzed with RT-qPCR.

In study **IV**, we analyzed the levels of 30 cytokines, chemokines, and growth factors from the DIABIMMUNE birth cohort samples with a customized 38-plex kit complemented with the Human High Sensitivity T Cell Panel - Immunology Multiplex Assay (Merck, Darmstadt, Germany). The samples from the young children's cohort were analyzed with the Human TH17 Magnetic Bead Panel - Immunology Multiplex Assay (Merck, Darmstadt, Germany) complemented with a custom 3-plex assay for EGF, sCD40L, and TGF-α. Samples from different countries were analyzed mixed on plates. Luminex analyses were performed in single reactions. Quantification of the markers was performed with the Bio-plex 200 Luminex instrument and Bio-Plex Manager software (Bio-Rad, Hercules, CA, USA). The concentration of each marker was determined from an eight-point standard curve using five-parameter logistic regression. The minimum detectable concentration (MinDC) was determined for each marker separately using the lowest concentration on the standard curves in the linear phase (MinDC = c(low)+2SD). The samples below MinDC were given a value of 50% of MinDC.

6.2.3 Serum and plasma 25-hydroxyvitamin D and 1,25-dihydroksyvitamin D measurements

The 25(OH)D concentration was measured from serum and plasma samples using an enzyme immunoassay (EIA) method (IDS OCTEIA 25-Hydroxyvitamin D kit [Immunodiagnostic Systems, Boldon, UK]). The intra-

and inter-assay coefficients of variation were 3.6% and 3.7% for study **I** and 3.1% and 1.4% for study **II**. Quality control was ensured by participating in the vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London, UK). Different Vitamin D concentrations were categorized as deficiency <25 nmol/l, insufficiency 25–49 nmol/l, sufficiency 50-75 nmol/l, and optimal >75 nmol/l in study **I** and <25 nmol/l, 25-50 nmol/l, 50-80 nmol/l, and >80nmol/l, respectively, in study **II**.

The $1,25(OH)_2D$ concentration was measured from plasma samples using a commercial EIA method (IDS 1,25-Dihydroxy Vitamin D EIA kit [Immunodiagnostic Systems, Boldon, UK]). The intra-assay coefficient of variation was 0.02%.

6.2.4 Amphiregulin ELISA

Undiluted plasma samples were analyzed for amphiregulin with the commercial Human Amphiregulin ELISA kit (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions.

6.2.5 HLA genotyping and analysis of diabetes-associated autoantibodies

The HLA genotyping of major type 1 diabetes-risk DR-DQ haplotypes of the study subjects in studies **II**, **III**, and **IV** was performed with a PCR-based lanthanide-labeled hybridization method using time-resolved fluorometry for detection, as previously described [230].

Specific radiobinding assays were used to quantitate diabetes-associated antibodies IAA, GADA, IA-2A and ZnT8A, as described elsewhere [231]. The cut-off levels used were 2.80 relative units (RU) for IAA, 5.36 RU for GADA, 0.78 RU for IA-2A, and 0.61 RU for ZnT8A, covering the 99 per-

centiles in more than 350 non-diabetic Finnish children. Islet cell antibodies were measured using an indirect immunofluorescence method with a cut-off value of 2.5 Juvenile Diabetes Foundation units. In study **II**, assays were performed three times at each study center visit and at least twice in study **III** to ensure persistent autoantibody formation. In study **IV**, only subjects negative for autoantibodies were included.

6.2.6 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

In studies **II** and **IV**, the total RNA from CD4+ memory cells and in study **III** the RNA from PBMCs was isolated with the Qiagen RNAeasy Mini Kit (Qiagen). The RNA from isolated Th17 cells in study **III** was extracted with the RNeasy Plus Micro kit (Qiagen). The random hexamer priming of a high-capacity cDNA reverse transcription kit was used to synthesize cDNA (Applied Biosystems, Foster City, CA). The StepOne Plus instrument and TaqMan Fast Master Mix (Applied Biosystems, Foster City, CA) were used to perform qPCR. The relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [232].

6.2.7 Metabolic parameters

The results regarding C-peptide levels, glucose concentrations in the oral glucose tolerance test, and HbA1c levels in study **III** were received from collaborators.

6.2.8 Stool sample collection, DNA isolation and microbiome analysis

Collection of fecal samples and DNA extraction in the birth cohort samples in study **IV** were performed as previously described [202, 233, 234].

16s sequencing and microbiome analysis was performed essentially as described in [233].

6.2.9 Statistical analyses

In study **I**, analyses were performed with PASW Statistics for Windows. The data were normally distributed and hence the Student's *t*-test was used in study **I** to compare 25(OH)D concentrations. To analyze the proportion of cases and controls in different vitamin D level groups, Pearson's χ^2 test was used.

The statistical analyses in studies **II**, **III**, and **IV** were performed with GraphPad Prism 4.03 to 8.30 software (GraphPad, La Jolla, CA), with the exception of correlation analyses, which were performed with SPSS 20 to 25 software (IBM, Chicago, IL). In study **II**, normally distributed data were analyzed with one-way ANOVA and the unpaired Student's *t*-test. The non-parametric Kruskal-Wallis test, Dunn's test and Mann-Whitney *U*-test were used for comparisons between groups in the studies. The non-parametric Spearman test was used to study correlations. In study **III**, $\log_{10}$-transformed data were analyzed with one-way ANOVA and the unpaired or paired Student's *t*-test. The two-tailed Fisher's exact test was used to analyze dichotomized data. In study **IV**, longitudinal analysis of the serum cytokine levels in different countries was performed with a linear regression model. The relationships between different taxa of gut bacteria and circulating cyto-, chemokines, and growth factors were analyzed with a linear mixed effects model, correcting for age, country of origin, and random effects (process batch and subject ID). *P*-values < 0.05 were considered significant in all studies. The *P*-values for differences were not corrected for multiple testing, except for the analysis of the relationship between the

gut microbiome and circulating cytokines in study **IV**, where the Benjamin-Hochenberg method was used.

7 Results and discussion

7.1 No differences in 25(OH)D concentrations during the first trimester of pregnancy in mothers with healthy children compared to mothers with children who later developed type 1 diabetes (I)

Maternal vitamin D deficiency and its role in fetal development has been assessed in numerous studies. One study determined that a low maternal vitamin D intake via food was associated with a higher risk of islet autoimmunity in the offspring [235]. Another study observed that the maternal intake of cod-liver oil reduced the risk of the offspring developing type 1 diabetes either due to vitamin D or the n-3 fatty acids eicosapentaenoic acid or docosahexaenoic acid [236]. In one study, published shortly after ours, lower serum 25(OH)D concentrations during late pregnancy were in fact associated with the risk of the child developing type 1 diabetes [237].

Our study investigated whether the vitamin D status during the first trimester of pregnancy was associated with the risk of type 1 diabetes in the offspring. Serum samples from mothers with healthy children and mothers with type 1 diabetic children were compared and no differences were observed. The mean 25(OH)D concentration during pregnancy was 43.9 nmol/l for the case mothers (n = 343; SD 16.9) and 43.7 nmol/l for the control mothers (n = 343; SD 16.6; p = 0.70). The samples available from pregnancies with a healthy sibling (n = 137) showed no differences compared to samples from pregnancies with a type 1 diabetic child when calculated using a season-standardized mean (44.1 nmol/l vs. 44.7 nmol/l; p = 0.60). No differences were seen in the proportions of case and control mothers in different 25(OH)D groups (p = 0.88). There was no difference in the 25(OH)D concentration when groups stratified by disease onset were

compared (0–3 years, 44.0 nmol/l; n = 177 vs. 4–7 years, 43.9 nmol/l; n = 154, p = 0.96).

The serum 25(OH)D concentration is known to be affected by seasonal differences in sunlight exposure. In this study, case and control samples were matched to be collected on the same date. Even though 25(OH)D is quite stable during storage, there might have been some degradation. This was evaluated in our study by measuring samples that had been stored for different times, and no difference was observed in the mean concentrations. Another study measured two year-old samples from the Finnish Maternity Cohort and also measured low concentrations (35.7 and 54.2 nmol/l in the first trimester and 40.5 and 59.3 nmol/l postpartum) with the same method in the same laboratory [238]. Despite the difference in maternal 25(OH)D concentrations, the concentrations in children at the age of 14 months were similar (63.0 vs. 66.0 nmol/l). This indicates that the effect of maternal 25(OH)D concentrations might not be as long-lasting.

The results of our study indicate that a decreased 25(OH)D concentration during the first trimester of pregnancy is not a risk factor for type 1 diabetes in the child. A study by Miettinen et al. revealed that two VDR SNPs linked with 25(OH)D rather than 25(OH)D concentrations were associated with the risk of the child developing type 1 diabetes [239].

However, during later pregnancy, 25(OH)D concentrations might influence the risk of developing type 1 diabetes, as observed in a study published after ours [237]. The authors reported that mothers of children with type 1 diabetes had lower 25(OH)D concentrations than mothers with healthy children during the last trimester of pregnancy (65.8 nmol/l vs. 73.1 nmol/l). The storage time of the samples was similar to our study (samples taken during 1992–1994), and these samples were also matched for seasonal changes.

Moreover, the amount of sunlight exposure is similar in Finnish and Norwegian women due to the similar latitudes (60° N for Oslo and Helsinki). However, the methods used differed between these studies and the assay CV% values were lower in our study (EIA vs. RIA). The recommendations for vitamin D intake in the 1990s were similar in Norway and Finland (5 µg/d) [240, 241]. The main vitamin D source in Norway is fatty fish, fortified margarine and butter, and cod-liver oil supplements, while the main sources in Finland are fish and fish products, fortified milk products, and vitamin D supplements [242]. It has been shown that 40% of the population in Norway regularly uses cod-liver supplements, almost 60% consumes fish liver products, and 57% other vitamin D supplements [243]. Cod-liver supplements are problematic as vitamin D sources, since they also contain n-3-polyunsaturated fatty acids. It might be that cod-liver oil is a much more efficient source of overall vitamin D intake, since supplements contain less vitamin D than oil, and this might have led to the higher 25(OH)D concentration observed in the Norwegian study of Sorensen et al. 2012 [237]. Another possibility is that the 25(OH)D obtained from supplements in Finland acts metabolically differently in modulating the immune system. The main caveat in our study is the fact that all the mothers had low 25(OH)D concentrations and therefore any differences in effects on the offspring were difficult to observe. A follow-up sample from the last trimester or a cord-blood sample could have provided more information, and possible differences in concentrations between the mothers could have been detected. One recent study demonstrated that 25(OH)D concentrations vary significantly during pregnancy [244]. The total 25(OH)D concentration was lowest during the first trimester, even though it was also low during the following two trimesters (median around 40 nmol/l in both). Recently,

it was observed that a low 25(OH)D concentration during the first trimester was a risk factor for general adverse pregnancy outcomes [245].

To conclude, Finnish mothers have low 25(OH)D concentrations during the first trimester of pregnancy, but this is not associated with the risk of type 1 diabetes in the offspring. Others have reported that low 25(OH)D concentrations during the last trimester are associated with the type 1 diabetes risk in the offspring. This might also have been seen in our study if follow-up samples had been available. Therefore, we cannot completely exclude the possibility that vitamin D deficiency later during pregnancy is a risk factor for the child developing type 1 diabetes and might contribute to the high incidence of type 1 diabetes in Finnish children.

7.2 Comparison of vitamin D status and active hormone form of vitamin D in Finnish and Estonian children with or without signs of beta-cell autoimmunity and its impact on CD4+ T memory cells (II)

Studies on the intake of vitamin D and the risk of beta-cell autoimmunity or type 1 diabetes have suggested a protective role for vitamin D [192, 236, 246]. However, these types of studies do not tell the whole truth regarding the role of vitamin D due to sunlight exposure. Therefore, it is important to measure the body's whole vitamin D content with 25(OH)D measurements. Data on 25(OH)D concentrations in newly diagnosed type 1 diabetes patients are inconsistent [247-250]. Vitamin D has been considered as an anti-inflammatory factor, and has several target genes in immune cells. In T helper cells alone, one study identified 102 target genes that were regulated by vitamin D [251]. Recently, it was shown *in vitro* that vitamin D induced human Treg differentiation and TGF-β1 secretion and inhibited Th17 differentiation and IL-17 secretion [252]. Since T cell aberrancies have been

associated with type 1 diabetes, we wanted to study the possible effects of circulating vitamin D concentrations on T cell function at the transcriptional level. One study noted that there was no association between either the vitamin D intake or 25(OH)D concentration throughout childhood and the risk of beta-cell autoimmunity or type 1 diabetes [253].

We aimed to investigate whether there are differences in 25(OH)D concentrations between Finnish and Estonian children with or without signs of beta-cell autoimmunity due to the much lower incidence of type 1 diabetes in Estonia compared to Finland. Estonian children had much lower 25(OH)D concentrations than Finnish children (Figure 2A, mean 48.4 nmol/l vs. 74.5 nmol/l, $p < 0.001$), and this was seen throughout the year (Figure 2B).

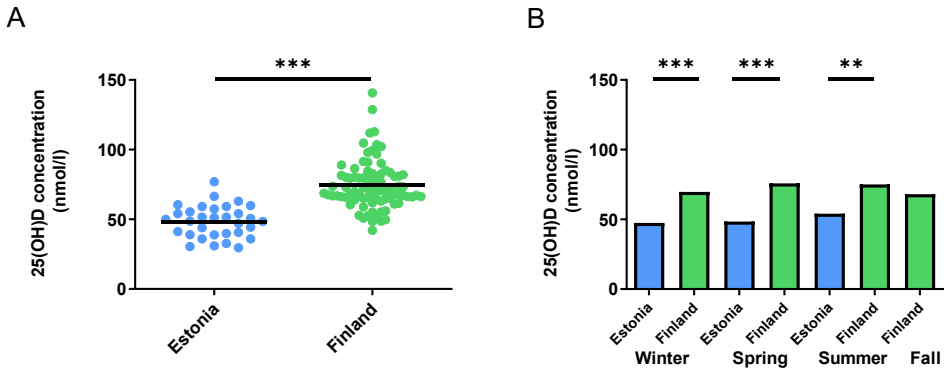

Figure 2. (A) Plasma 25(OH)D is significantly higher in Finnish children (***$p < 0.001$). (B) Seasonal variation in 25(OH)D concentrations in both countries. Horizontal lines represent means (***$p < 0.001$, **$p < 0.01$).

There were no differences in 25(OH)D concentrations between children with and without signs of beta-cell autoimmunity (70.6 nmol/l vs. 65.7 nmol/l). When children with multiple autoantibodies were separated as one group, the results remained the same (Aab-: 65.7 nmol/l; 1 Aab: 65.0

nmol/l; >1 Aab: 73.2 nmol/l). These results are in line with one previous study [253].

Interestingly, the expression of FOXP3 was elevated in Estonian CD4+ T memory cell samples (56.2 vs. 42.8, $p < 0.01$). In order to determine whether this was due to a higher 25(OH)D concentration in certain individuals, we separately analyzed only samples having sufficient 25(OH)D concentrations from both countries (50–80 nmol/l). The expression of FOXP3 remained higher ($p < 0.001$) in the Estonian population. In order to determine whether vitamin D conversion was locally different, we investigated the expression of the vitamin D3 convertase gene *CYP27B1* in CD4+ T memory cells. Here, we did not find any differences between countries or study groups. However, it should be noted that the amount of functional enzyme or enzyme activity of vitamin D3 convertase was not investigated. To examine whether the conversion of 25(OH)D into the active hormone $1,25(OH)_2D$ was a limiting factor in our study, we extracted and measured the active hormone from a small subcohort (N = 56). No differences were detected between the countries (Estonia: 100.6 pmol/l; Finland: 96.4 pmol/l), or between different autoimmunity groups (Aab-: 98.7 pmol/l; 1 Aab: 97.4 pmol/l; >1 Aab: 100.2 pmol/l). Even though Estonian children had lower 25(OH)D concentrations, they had the same sufficient amount of active hormone in the blood compared to Finnish children. On the other hand, it is known that $1,25(OH)_2D$ is strictly regulated and hence differences in its concentration are difficult to observe.

In conclusion, there were no differences in the vitamin D status or levels of the active hormone form between children with or without signs of beta-cell autoimmunity, suggesting that vitamin D is not a protective factor against beta-cell autoimmunity. Estonian children had lower 25(OH)D concentrations than Finnish children, but their $1,25(OH)_2D$ concentrations

were at the same sufficient level. Finnish children had nearly optimal vitamin D levels (~75 nmol/l), whereas Estonian children had insufficient levels (below 50 nmol/l). This suggests that the amount of $1,25(OH)_2D$ needed for normal health is obtained without vitamin D supplementation. The excess 25(OH)D in Finland could potentially even have some negative effects. This also leads to the question of whether vitamin D obtained from supplementation really has any impact on metabolic conversion and function in the body. Estonian children had higher FOXP3 mRNA expression in their CD4+ memory cells, which suggests that the self-regulation in their CD4+ T cells is intact and possibly affected by some environmental agents that have disappeared from the environment in Finland. No correlations between vitamin D and cytokines expressed in CD4+ memory cells were observed.

7.3 Th17 immunity and the plasticity of Th17 helper cells in the development of type 1 diabetes (III)

The role of Th17 cells in the development of type 1 diabetes has previously been suggested in both animal [127] and human studies [21, 129-131]. One study also detected elevated plasma levels of IL-17 in children with autoantibodies compared to autoantibody-negative children [254]. The role of plasticity in Th17 helper cells has previously been implicated in animal studies in which Th17 cells from BDC2.5 mice induced autoimmune diabetes in healthy recipients after conversion into Th1 cells [134, 135]. Potential plasticity in human Th17 cells has been reported before in patients with type 1 diabetes. Marwaha et al. [129] demonstrated that Th17 cells also expressed FOXP3, even though they originally aimed to investigate Treg plasticity. Beriou et al. [255] observed IL-9 expression in Th17 cells from type 1 diabetes patients.

The aim of our study was to determine the changes in Th17 immunity during the development of type 1 diabetes. We were also interested in measuring the potential plasticity of Th17 cells. We observed that PBMCs from children with advanced signs of beta-cell autoimmunity and impaired glucose tolerance (IGT) displayed upregulation of Th17 immunity (Figure 3).

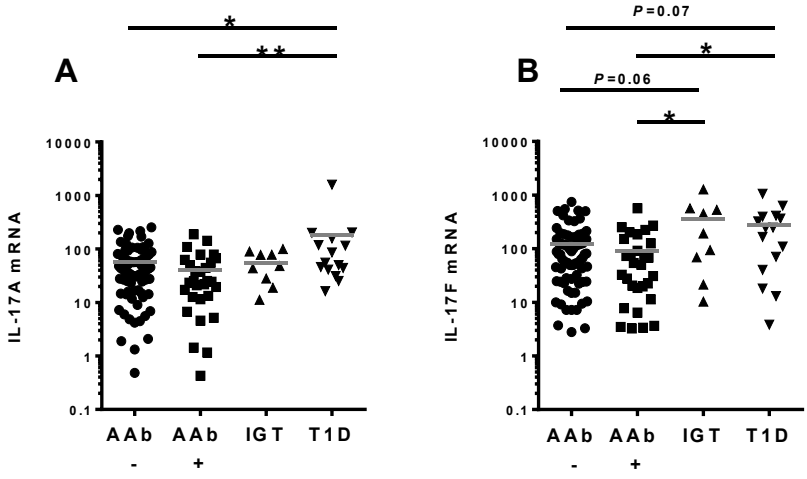

Figure 3. Children with advanced beta-cell autoimmunity and impaired glucose tolerance or type 1 diabetes show an up-regulation of IL-17 immunity (*$p < 0.05$, **$p < 0.001$) in PBMCs stimulated for 40 h. (A) Relative mRNA expression of IL-17A, (B) relative mRNA expression of IL-17F.

Upregulation of IL-9 and IFN-γ was also observed in PBMCs of these individuals (Figure 4). The upregulation of Th1 responses has been reported in type 1 diabetes before, and Th17 cells converting to a Th1 phenotype have been implicated to be especially pathogenic [123, 135]. IL-9-secreting Th17 cells induced by TGF-β have previously been observed in type 1 diabetes [255]. This could suggest that the T helper cell phenotype in the children we studied was more plastic and prone to converting into a more pathogenic phenotype.

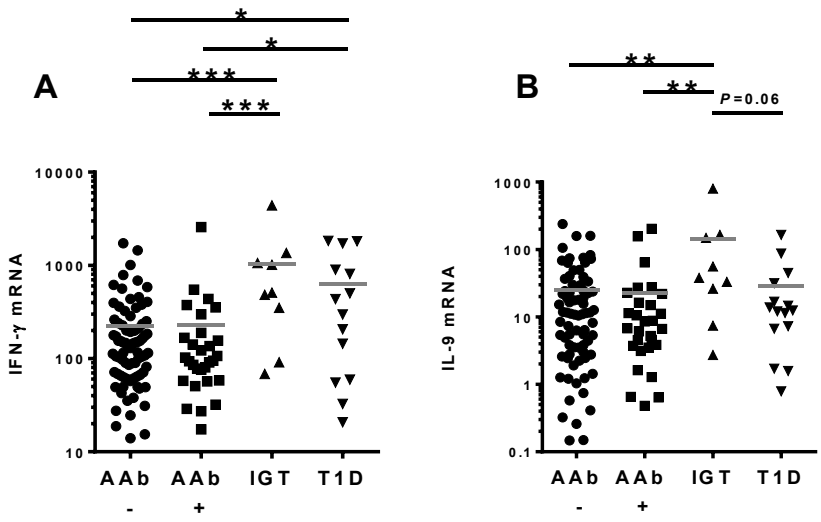

Figure 4. Children with advanced beta-cell autoimmunity and glucose intolerance have higher IFN-γ (A) and IL-9 (B) mRNA expression in their PBMCs (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

In order to further evaluate the plasticity of Th17 cells, the cells were sorted and their gene expression profile was investigated. The gating strategy is illustrated in Figure 5.

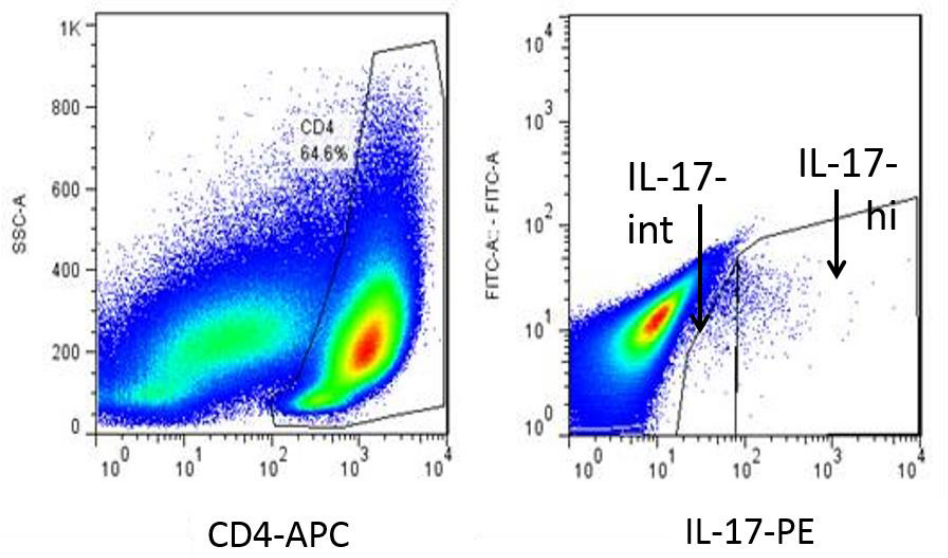

Figure 5. Identification of IL-17-secreting T helper cells for FACS analysis and sorting. Lymphocytes were first identified in the light scatter and gated further for CD4 and IL-17. Purified cells were subjected to RT-qPCR analysis of IL-17A, IL-17F, IFN-γ, and IL-9.

We gated cells with a strict IL-17 high gate to ensure purity of these cells, but also investigated the population of intermediate IL-17 producers. To investigate the amount of plasticity in these cells, we used the amount of IFN-γ mRNA expression divided by the IL-17 mRNA expression as a plasticity index. We observed that children with advanced beta-cell autoimmunity and IGT had elevated IFN-γ/IL-17 ratios, i.e. plasticity indices, in both Th17int cells and Th17high cells (Figure 6). This suggests that they potentially harbor Th17 cells converting into a Th1/Th17 phenotype close to disease diagnosis. These results are in line with previous findings from animal studies [134, 135]. We did not find any evidence that Th17 cells originated from thymic-derived Tregs, because the expression of Helios, a marker of thymic-derived Tregs, was low in sorted Th17 cells and did not differ between the groups. Also, the expression of FOXP3 was similar in Th17 cells from all groups. Moreover, Kunicki et al. demonstrated that the

percentage of Th17 cells overlapping with Treg cells in their analysis was rather low (3.04%) [256].

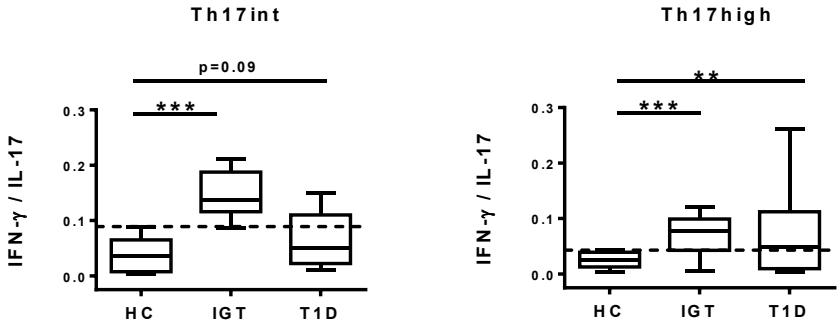

Figure 6. Children with advanced beta-cell autoimmunity and glucose intolerance show higher IFN-γ/IL-17 ratios in the sorted Th17int cells and Th17high cells compared to healthy children (***$p < 0.001$). The IFN-γ/IL-17 ratio is also higher in Th17high cells of type 1 diabetes children compared to healthy controls (**$p < 0.01$).

The Th17 plasticity index also correlated with both HbA1c levels and plasma glucose concentrations in the oral glucose tolerance test, reflecting the function of beta cells (Table 5).

Table 5. The ratio of IFN-γ/IL-17 from Th17high cells correlated with the fasting glucose concentration, glucose concentration after the oral tolerance test, and HbA1c.

	IFN-γ/IL-17
Glucose mmol/l (0 h)	r = 0.81, p = 0.05
Glucose mmol/l (2 h)	r = 0.94, p < 0.01
HbA1c	r = 0.89, p < 0.05

Our results suggest that the Th17/Th1 plasticity in the periphery is a phenomenon of advanced autoimmunity and glucose intolerance in the disease development process for type 1 diabetes. It has been shown in mice that Th17 cells are present early but remain inactive [257]. The mechanisms

behind Th17 activation were not investigated in our work, but the gut microbiota is considered to be an essential factor in the regulation of mucosal and peripheral immunity. It has been noted before that *Candida albicans* induces IL-1β from monocytes, which results in IFN-γ and IL-17 secretion from Th17 cells, while *Staphylococcus aureus* induces IL-17 and IL-10 secretion from Th17 cells [139]. This could suggest the involvement of these microbes in our study samples, and it would be interesting to examine the relationship between alterations in the gut microbiota and the activation of Th17 immunity. Liu et al. observed that TGF-β- and IL-6-induced Th1 cells convert to Th17 cells through Runx1 in the gut of a mouse model of colitis [258].

In mice models, it has been seen that colonization of the gut with SFB resulted in the differentiation of antigen-specific Th17 cells in the small intestine [259-261], and that this resulted in enhanced resistance to the bacterium *Citrobacter rodentium* [259]. Wu et al. reported that SFB-induced Th17 cells were able to trigger autoimmune arthritis in mice [262]. However, few studies have addressed these phenomena in humans. A study by Atarashi et al. revealed that bacterial strains needed to adhere to epithelial cells in order to induce Th17 cells [263]. From one fecal sample from a patient in the US, they isolated 20 strains, most of them belonging to *Clostridium, Bifidobacterium, and Ruminococcus* species, that were able to induce Th17 cells in mice. In a study by Tan et al., the human symbiont *Bifidobacterium adolescentis* was able to induce Th17 cells in the gut of mice and to exacerbate autoimmune arthritis [264]. The authors further observed that IBD patients had more *Bifidobacterium*-related species with a high abundance in their gut compared to healthy control data from the MetaHIT database. They also tested six different off-the-shelf probiotic products and found that four of them were able to induce intestinal Th17 cells

in mice [264]. Vatanen et al. [234] compared the microbiome of children from Finland, Estonia, and Russia. They found that even though Finnish and Estonian children were breastfed longer than Russian children, the genus responsible for metabolizing human milk oligosaccharides (HMOs) was in fact *Bacteroides* in Finland and *Bifidobacterium* in Russia [234]. They noticed that one possible mechanism contributing to an increased risk of autoimmunity in Finland and Estonia could be that the LPS derived from *Bacteroides* did not induce endotoxin tolerance compared with *E. coli*-derived LPS [234]. We have also observed that *Bifidobacterium longum* negatively correlates with Th17 immunity in healthy controls (unpublished results). This is an interesting aspect to be investigated further and it could raise a question of whether off-the-shelf probiotic products should be recommended for individuals at risk of autoimmune diseases.

In MS patients, it has been reported that vitamin D supplementation increases serum TGF-β [251]. If TGF-β induces Th1 cells to convert to Th17 cells [258] and TGF-β inhibits IL-22 in Th17 cells [41], this might lead to more pathogenic cells in Finland, where vitamin D supplementation is commonly implemented. Previously, it has also been observed that Finnish mothers have elevated TGF-β levels in their breast milk compared to mothers from other European countries [265], and hence this priming of T cells might already start during breastfeeding.

The Th17 memory cell population and IL-17A concentration in serum have been reported to be much higher in MS patients and neuromyelitis optica (NMO) patients compared to healthy controls [266]. Annunziato et al. also demonstrated the presence of Th17 and Th1/Th17 cells in the gut of patients with Crohn's disease [267].

Results regarding Th17/Th1 plasticity in other autoimmune diseases are similar to our findings. Comparable Th1/Th17 plasticity has been observed

in CD4+CD45RA+ cells of patients with autoimmune polyendocrinopathy-candidasis-ectodermal dystrophy (APECED) compared to controls [268]. Already in 2010, Nistala et al. demonstrated that Th17 cells in the inflamed joints of children with inflammatory arthritis expressed a Th1 phenotype and converted into Th17/Th1 (RORC- and T-bet-expressing) cells under low TGF-β and high IL-12 concentrations [136]. Kotake et al. reported that Th17 cells present in the peripheral blood of early RA patients were derived from Th1 cells (CD161+ Th17 cells) [269]. They stated that anti-IL17 antibodies could potentially be therapeutically effective in patients with early RA. Basdeo et al. detected ex-Th17 cells that converted to Th1 cells in the inflamed joints of RA patients and observed that these cells secreted more proinflammatory cytokines upon stimulation and were resistant to suppression of Tregs [270]. This might also be true in the preclinical phase of type 1 diabetes. It is possible that these highly inflammatory Th17 cells are the first ones in close proximity to the pancreas and appear in the circulation close to the diagnosis. Since we could not see any upregulation of FOXP3 in the Th17/Th1 cells in children with impaired glucose tolerance, it might support the idea that these cells are resistant to self-regulation.

To conclude, children with advanced beta-cell autoimmunity express signs of Th17 immunity, as has been observed in type 1 diabetes patients before. Stimulated PBMCs from children with advanced beta-cell autoimmunity express higher IL-17F, IFN-γ, and IL-9 mRNA levels, and higher IFN-γ expression was observed in sorted Th17int and Th17high cells from these children. Importantly, the IFN-γ/IL-17A mRNA ratio in Th17high cells correlated with metabolic parameters, suggesting that Th17 immunity close to disease diagnosis and the plasticity of these cells potentially accelerates

disease development due to the gain of the Th1 phenotype fueling cytotoxicity. This is a novel finding and these markers may be used in the identification of disease progressors among children with beta-cell autoimmunity. The use of ROR inhibitors as therapeutic agents has been shown to be effective in a NOD mouse model [141]. In that study, a RORα/γ inverse agonist, SR1001, was used and this reduced diabetes incidence and insulitis in the treated mice. SR1001 reduced proinflammatory cytokine expression, especially that of Th17-related cytokines, reduced autoantibody production, and increased the frequency of Tregs [141]. Xiao et al. identified three inverse agonists for RORγt (TMP778, TMP920, and GSK805) that were able to suppress Th17 responses and disease in EAE mice [271]. In psoriasis and psoriatic arthritis, there are already FDA-approved antibodies targeting Th17 immunity [272-275]. We did not test the antigen specificity of the Th17 cells. This would have been very interesting and is something to consider in future studies. On the other hand, *in vitro* experiments have demonstrated that IL-17A together with IL-1β and IFN-γ increase the frequency of apoptotic beta cells and increase the beta-cell stress response, suggesting that beta-cell death may be a bystander effect without antigen specificity.

In conclusion, the up-regulation of Th17 immunity and the plasticity of Th17 cells is not associated with the early stages of type 1 diabetes disease development, but rather with advanced disease pathogenesis. These can potentially be used as a biomarker for disease progression and as a tool for identifying high-risk individuals for inclusion in secondary intervention trials and for immune monitoring in these trials. The upregulation and plasticity of Th17 immunity may be a result of alterations in the microbiota on mucosal surfaces, such as the gut, where *Candida* and some bacteria could be the potential drivers.

7.4 Mucosal immunity in young Russian, Estonian, and Finnish children – focus on epidermal growth factor and interleukin-22 (IV)

Environmental factors modulate the development of the immune system and influence the risk of autoimmune and other immune-mediated diseases, as seen in differences in the incidence of allergies, asthma, and autoimmune diseases between Finnish, Estonian, and Russian Karelian populations [276-279].

To elucidate some of the molecular drivers for this difference, we investigated the development of the immune system in infants participating in the DIABIMMUNE study during the first three years of life in Russian Karelia, Estonia, and Finland by measuring the levels of 30 cytokines, chemokines, and growth factors in serum samples. Additionally, we validated the results from the first phase by analyzing these further in an independent set of samples from four-year-old children from Estonia and Finland.

We observed a clear increase in blood levels of circulating cytokines associated with Th1 (IFN-γ), Th2 (IL-5 and IL-13), Th17 (IL-17A), and regulatory T cell (IL-10) subsets in all countries during the first three years of life. For all the Th cytokines listed above and for the cytokines supporting the development of different Th subsets, age was a significant driver, whereas we did not observe differences between the countries in the longitudinal analysis. The maturation profile was similar between the countries. Analysis of the birth cohort samples for growth factor, cyto-, and chemokine levels revealed that Russian children had significantly increased circulating levels of EGF, sCD40L, and CXCL11 (ITAC) compared to children from Estonia and Finland during the study period. As we noticed differences in the levels of EGF and TGF-α between the countries in the birth cohort, we decided to include AREG in our analyses to examine whether

there is any specificity in the EGFR ligand-level differences. When we examined concentrations of EGF, TGF-α, AREG, and sCD40L in four-year-old children in Estonia and Finland, we observed that Estonian children had elevated levels of EGF and sCD40L compared to Finnish children ($p < 0.001$). In turn, Finnish children had higher concentrations of TGF-α than Estonian children ($p < 0.001$). No difference was observed in AREG concentrations.

Both EGF and TGF-α are high-affinity ligands for EGFR, causing proliferation and differentiation of their target cells [280]. Despite similar affinities, they possess a different potency to activate EGFR-mediated signaling, with TGF-α being more potent [281, 282]. Upregulation of TGF-α in Finnish children could be an adaptive mechanism to compensate for the lower EGF levels. However, since EGF and TGF-α activate slightly different signaling cascades and modify the turnover of internalized EGFR-ligand complexes differently [281], the altered levels in Finnish children most likely modify the function of the cells expressing EGFR, including intestinal epithelial cells.

We next analyzed the relationship between EGF and sCD40L, and observed that in Estonia and Finland, these factors showed a strong correlation throughout the study period (Table 6). In Russia, a similar correlation was only detected in six-month-old children. In children from the young children's cohort, we observed a strong correlation in both Estonia and Finland ($r = 0.69$, $p < 0.001$ and $r = 0.58$, $p < 0.0001$).

Table 6. The correlation of EGF and sCD40L levels at different ages in Russia, Estonia and Finland during the three-year study period.

	3 months	6 months	12 months	18 months	24 months	36 months
Russia	NS	r = 0.69, $p < 0.0001$	NS	NS	NS	NS
Estonia	r = 0.72, $p < 0.0001$	r = 0.4, $p < 0.05$	r = 0.65, $p < 0.01$	r = 0.65, $p < 0.01$	r = 0.84, $p < 0.0001$	r = 0.5, $p < 0.05$
Finland	r = 0.8, $p < 0.001$	r = 0.5, $p < 0.001$	r = 0.48, $p < 0.01$	r = 0.5, $p < 0.05$	NS	r = 0.74, $p < 0.0001$

When we further analyzed samples from the young children's cohort, we observed that the concentrations of circulating IL-17A and IL-6 were higher in Finnish than in Estonian samples ($p < 0.01$). The concentrations of IFN-γ and IL-12p70 were also higher in Finnish samples compared to Estonian samples ($p < 0.01$). However, the protein concentrations of IL-22 and IL-1β were at the same level in both countries. These results suggest that environmental factors in Finland have changed towards a Th1/Th17 direction, potentially promoting the development of immune pathologies.

As EGF was consistently higher in Russian and Estonian children, we aimed to characterize the relationship between effector pathways and EGF in more detail. When we divided the children from the young children's cohort into EGFlo and EGFhi groups based on the overall median value, we observed that Finnish children expressing high levels of EGF had higher levels of CCL20 and IL-13 compared to Estonian low and high EGF expressors and Finnish low EGF expressors ($p < 0.05$ to $p < 0.01$). Estonian EGF low expressors had lower IFN-γ mRNA expression in CD4+ memory cells than Estonian high EGF expressors and Finnish children with low or high expression of EGF ($p < 0.05$ to $p < 0.01$). Previously, it has been noted that the EGF receptor is under regulation by IFN-γ [283]. It appears that in

Estonia there is a link between IFN-γ and EGF, but this link has been lost in Finland. In the Finnish EGFlo expressors, we detected a modest correlation between TGF-α and IFN-γ mRNA expression in CD4+ memory cells, further supporting the replacement of EGF by TGF-α in Finland ($r = 0.371$, $p < 0.05$). Furthermore, there appears to be a connection between the levels of circulating EGF and CCL20 and IL-13 in Finland, suggesting that EGF may modulate T cell functions more broadly. Kim et al. observed that CCL20 indirectly activated the EGF receptor, and this led to the exaggerated production of MUC5AC mucin from human airway epithelial (NCI-H292) cells [284]. Interestingly, TGF-α also induced activation of ERK 1,2-dependent production and secretion of CCL20 from airway epithelial cells. We observed higher TGF-α levels but lower EGF levels in Finnish children. Finnish children with high EGF had higher CCL20 levels compared to Finnish children with low EGF or Estonian children in general, suggesting that in Finland, a functional switch between EGF and TGF-α has occurred. Therefore, the consequence of altered levels of EGF and TGF-α in Finnish children could be functional alterations of mucosal immunity. CCL20, the only known ligand for CCR6, is expressed at high levels on Th17 cells, regulating the recruitment and migration of these cells in the gut-associated lymphoid structures [285]. Chen et al. reported that IL-22 was able to inhibit the secretion of CCL20 in *Helicobacter pylori*-infected epithelial cells *in vitro*, and an inverse association was also seen in tissue samples from patients with *H. pylori* infection [286]. Increased secretion of mucins and CCL20 could alter the capture of antigens and migratory patterns of mucosal immunity-associated Th17 cells in the gut mucosa.

IL-13 has also been linked with EGFR. Zhen et al. observed that CCL20 and IL-13 both influenced the production of mucus in airway epithelial

cells with distinct pathways [287]. Despite sporadic observations of EGFR expression on hematopoietic cell lines, the expression and functional significance of EGFR in immune cells has only recently been recognized. In a study by Minutti et al., T cell receptor-induced expression of EGFR and ligation of AREG was essential for IL-33-induced secretion of IL-13 from activated Th2 cells in mice [288]. A recent study reported that activated human T cells express EGFR and that the inhibition of EGFR signaling decreased cell proliferation and the production of Th1/Th2/Th17 cytokines [289]. In a study by Zaiss et al., AREG, a mast cell-derived ligand with low affinity for EGFR, significantly enhanced Treg functions *in vivo* and *in vitro* [290]. These data suggest that EGFR signaling may be an important direct regulator of T cell functions in humans and requires more investigation in the future.

When we analyzed the mRNA expression of T helper memory cells in samples from the young children's cohort, we observed that the only marker that differed between countries was IL-22, as it was significantly higher in Estonian children than Finnish children ($p < 0.001$). There was no difference in IL-17A, IFN-γ, or FOXP3 mRNA expression between the countries.

IL-22 is a crucial regulator of intestinal immunity, inflammation, and tissue repair. The IL-22 receptor is widely expressed in non-immune cells, but is absent from immune cells, and IL-22 mainly influences epithelial cells, hepatocytes, pancreatic acinar cells, and related stem cells [291]. IL-22 induces the secretion of anti-microbial peptides, such as β-defensin-2 from gut epithelial cells. IL-22 is important in protective immunity against *Citrobacter rodentium* in mice. Zheng et al. demonstrated that IL-22 knockout mice died from *Citrobacter rodentium* infection, and histological

samples from these mice displayed increased mucosal hyperplasia and submucosal inflammation compared to wild-type mice [214]. One study reported results from *in vitro* experiments in which the stimulation of keratinocytes with the supernatant of Th22 cells or recombinant IL-22 and TNF-α successfully inhibited *Candida albicans* infections and preserved epithelial health [215]. Since it has been proposed that the role of IL-22 is protective in acute inflammation and detrimental in chronic inflammation, the results suggest that chronic inflammation in Finland could support the protective role of IL-22 in Estonia and a more inflammatory role in Finland [292]. Previously, it has been observed that CD4+ memory cells that produce IL-22 can be *Candida albicans*-specific [293]. Possible yeast infections and urinary tract infections by *E. coli* could explain the IL-22 expression in memory cells in Estonian children. So far, this has not been investigated. One approach to further study this aspect would be to analyze the mycobiota composition of these children. One recent study observed that the onset of autoimmunity in a NOD mouse model was associated with modifications to the structure of the mucus layer and the loss of gut barrier integrity [294]. These mice carried a transgenic TCR specific for a beta-cell autoantigen, and it was observed that the loss of integrity led to the activation of islet-reactive T cells within the gut mucosa and subsequently to the onset of diabetes [294]. If the integrity of the intestinal epithelium is damaged to some extent in Finland, this could explain the up-regulation of cytokines essential for mounting cytotoxic responses against intracellular pathogens.

In this study, we only observed a few longitudinal differences between the countries, EGF showing the most striking differences. While the gut microbiota is considered an important regulator of peripheral immunity, we did not observe any significant correlations between different gut bacterial

taxa and the levels of circulating cytokines, chemokines, or growth factors. Very recently, Ruohtula et al. reported that the gut microbiota greatly influences the maturation of circulating regulatory T cells in early life when Estonian and Finnish children were compared [295]. In a study by Schirmer et al., the authors found that gut microbial signatures correlated with immune responses from stimulated PBMCs [296]. Here, we analyzed the levels of circulating soluble factors, thus presenting sliding average values at a given time and making it difficult to establish a link between the gut microbiota and circulating cytokines. On the other hand, there might be other factors affecting the regulation of EGF levels in the systemic circulation. One example of such a factor has been reported in a recent study in which vitamin D supplementation was demonstrated to decrease EGF expression in type 1 diabetes patients [297]. It was further shown that $1,25(OH)_2D$ alters membrane trafficking and downregulates growth signaling of the EGF receptor [298].

In conclusion, our results suggest that lower concentrations of EGF in Finland could contribute to enhanced Th1 and Th17 immunity. We further suggest that decreased expression of EGF and sCD40L in Finnish children may contribute to the disruption of epithelial integrity and intestinal homeostasis, potentially mediated by the decreased and modulated activity of antigen-experienced memory T helper cells. This, in turn, may contribute to the development of systemic low-grade gut inflammation, resulting in elevated levels of inflammatory cytokines in the periphery and favoring the development of immune-mediated diseases. Environmental factors that reduce EGF levels in early infancy in Finland are yet to be revealed.

8 Conclusions

Study I. A low concentration of vitamin D during the first trimester of pregnancy is not associated with type 1 diabetes in the offspring. Overall, Finnish mothers have insufficient 25(OH)D concentrations during the first trimester of pregnancy. Results from other studies have shown that low vitamin D concentrations during the last trimester are associated with type 1 diabetes in the offspring, but the results of these studies must be interpreted with caution due to simultaneous consumption of fatty acids. Follow-up samples from a later stage of pregnancy or at the time of birth would also have provided more information in our study. It is possible that vitamin D deficiency later during pregnancy is a risk factor for the child developing type 1 diabetes. This could be one environmental factor contributing to the high incidence of type 1 diabetes in Finnish children. However, our current results do not indicate that a decreased vitamin D status during early pregnancy is a risk factor for the offspring developing type 1 diabetes later in life.

Study II. We did not observe differences in the vitamin D status or levels of the active hormone form between children with or without signs of beta-cell autoimmunity. Moreover, no differences were observed in the vitamin D convertase CYP27B1 expression in CD4+ T helper memory cells between different autoimmunity groups or between countries. Interestingly, Estonian children had lower 25(OH)D concentrations than Finnish children, but their $1,25(OH)_2D$ concentrations were at the same sufficient level. This may suggest that the amount of $1,25(OH)_2D$ needed is obtained with-

out additional vitamin D supplementation. Estonian children had higher expression of FOXP3 in their CD4+ memory cells, suggesting that factors other than vitamin D sustain the activity of regulatory mechanisms in T cells. In the light of the current study, vitamin D is not a protective factor against beta-cell autoimmunity or type 1 diabetes.

Study III. Upregulation of Th17 immunity and increased expression of IFN-γ in Th17 cells is associated with advanced beta-cell autoimmunity and disturbances in glucose metabolism. Th17 immune markers may have potential as novel biomarkers for predicting disease progression. Importantly, Th17 cells may also represent an attractive therapeutic target for the pharmacological inhibition of type 1 diabetes-associated immunopathology. Our results suggest that close to a type 1 diabetes diagnosis, Th17 immunity is increased, and the plasticity of these Th17 cells is a factor possibly leading to disease development due to their conversion to the Th1 phenotype.

Study IV. We demonstrated lower serum levels of EGF and sCD40L, and decreased IL-22 mRNA expression in CD4+ T memory cells in Finnish children, which may be associated with a disruption of gut epithelial integrity and decreased activity of antigen-experienced memory T helper cells in these children. This, in turn, may result in low-grade gut inflammation, generating elevated levels of inflammatory cytokines in the periphery and favoring the development of immune-mediated diseases in Finnish children compared to Estonian and Russian children.

8.1 Potential caveats of the studies

Study I. It would have been interesting to compare serum samples from different time points of pregnancy to gain more information on vitamin D concentrations throughout pregnancy.

Study II. The number of studied individuals was rather small. We could have included more samples from additional individuals to ensure that the study had sufficient power to detect subtle differences. We did not characterize the phenotype or function of specific T helper subsets, but rather analyzed the whole memory T cell population. Analyses of enzymatic activity and enzyme levels of vitamin D convertase would have given more information regarding vitamin D conversion.

Study III. Antigen specificity could have been tested on some of the sorted Th17 cells to gain more information regarding their role. We only studied a limited number of individuals, and a larger prospective longitudinal follow-up study is therefore warranted in the future.

Study IV. The characterization of the EGF receptor in T helper cells and the effect of EGF stimulation on T cell phenotypes and responses would have been an interesting aspect to study further. The potential of serum samples to induce EGFR signaling needs to be evaluated in future studies. Also, results regarding the mycobiota would have been interesting. Stimulation of PBMCs or human insulinoma cell lines with EGF and IL-22 could be an experiment to consider in the future.

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